Department of Health

Report on Health and Social Subjects

35

Guidelines for the Testing of Chemicals for Mutagenicity

Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment

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Preface

The awareness that some chemicals in the environment have the potential to bring about changes in human hereditary processes, and hence to affect the health of subsequent generations, led the Department to publish their Guidelines for the Testing of Chemicals for Mutagenicity in 1981. These were drawn up on the basis of recommendations of the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, which advises Central Government on the mutagenicity of chemical substances.

In view of the rapid development in the field it was now felt timely to review these guidelines. Members of the Committee, under the Chairmanship of Professor B Bridges, have therefore prepared a new document on the Testing of Chemicals for Mutagenicity. This completely revises the existing guidelines and furthermore covers the use of mutagenicity data to obtain information on carcinogenic potential. Consideration is given to the present knowledge on genetic processes and mechanisms which provide the scientific basis for genetic toxicology, and the use of such data in screening for carcinogenic potential. I am grateful to members of the Committee for their detailed consideration of this area, and for compiling this important and professional document; it will be of much value to all those involved in the evaluation of the mutagenic properties of chemical substances.

E D Acheson
Chief Medical Officer
Department of Health
April 1989

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1. Introduction: Background to revision of COM guidelines

1.1 The first guidelines issued by the COM for the testing of chemicals for mutagenicity were published in 1981 and represented the state of the art of mutagenicity testing at that time.

1.2 Since then the pace of development in this field has been rapid on many fronts particularly with respect to the further refinements and validation of the existing test methods, the development of new procedures and the elucidation of mechanisms. This has necessitated updating the guidelines to reflect these advances. In particular there is a need to provide industry with guidance on the Committee's current views on a strategy for investigating the mutagenicity of chemicals. In order to provide this up-to-date information the original guidelines have been subjected to major revision. The framework adopted is that used in the 1981 guidelines, with the exception described in the following paragraph.

1.3 The 1981 guidelines contained a chapter (Chapter 8) outlining the principles of the recommended test systems. It is now not considered necessary to give such information in view of the number of publications now available giving detailed information on critical aspects of such assays. Examples of such texts are listed in the bibliography (Appendix I).

2. Summary

2.1 Chapter 3 entitled 'DNA, Genes and Chromosomes' gives background information on present knowledge on genetic processes and mechanisms and the role of DNA, since this is the basis of genetic toxicology.

2.2 Consideration is then given in Chapter 4 to the implications of mutagenicity data for carcinogenicity. Since the publication of the initial guidelines there has been increasing interest in the use of mutagenicity studies for screening chemicals for potential carcinogenicity. This has been due, in part, to the availability of additional data on the validation of this approach, including the results of large international collaborative studies. Increasing knowledge on the mechanisms of the carcinogenic process, in particular the discovery that point mutation and chromosomal mutation can lead to activation of proto-oncogenes and to inactivation of tumour-suppressor genes, show that mutation is of fundamental importance in the carcinogenic process. This emphasises the need for the use of mutagenicity tests as pre-screens for potential carcinogenicity, and particularly the significance that can be placed on short-term *in vivo* tests and their interpretation in this context; this topic was not considered in detail in the 1981 guidelines.

2.3 Chapter 5 considers genetic and partly genetic diseases in humans, and describes their type, frequency and mutation rates. The same framework is used as in the initial guidelines, with updating where necessary. The data provided clearly indicate the important role of genetic disease in health detriment in humans.

2.4 Chapter 6 covers monitoring of human populations for mutational changes as in the 1981 guidelines. In addition, this chapter considers monitoring of populations for exposure to genotoxic agents, and for genotoxic effects in human cells, approaches which are becoming increasingly important as biological indicators of exposure to, and uptake of, potentially mutagenic compounds.

2.5 The recommended strategy for testing compounds for mutagenicity is outlined in Chapter 7. This is essentially based on a hierarchical approach. The first stage, *in vitro* screening, is designed to ensure a high probability of detecting mutagenic potential. This is followed by *in vivo* studies to ascertain whether any activity seen in Stage 1 can be expressed in the whole animal, although it is recognised that with certain compounds, for example, those to which relatively high dose exposure is expected, some *in vivo* data will always be necessary if only to ensure that any mutagenic metabolites are detected in cases where all the Stage 1 tests are negative. The third phase consists of germ-cell assays. The germ-cell assays are required in order to obtain data for assessing the risks of heritable effects. These assays are divided into those which indicate potential, and assays that actually measure heritable effects in the offspring. The latter studies, which are expensive and which use large numbers of animals, can be justified only when quantitative data are required for a full human risk analysis. They are however the only approaches which provide quantitative data for such risk assessment.

2.6 In addition consideration is given in Chapter 7 to the use of mutagenicity data in pre-screening for carcinogenicity; the place of 'cell transformation', assays is also considered in this context.

2.7 A bibliography, together with an extensive glossary, is provided in two appendices at the end of the guidelines.

3. DNA, genes and chromosomes

Introduction

3.1 The structure and functioning of a living cell depends upon the presence and activity of a variety of macromolecules which include proteins. These macromolecules catalyse the chemical reactions necessary for cellular activity, provide structural rigidity, control the permeability of membranes and regulate other diverse tasks in the cell. Proteins are polymers (polypeptides) composed of specific arrangements of 20 different monomeric subunits, the amino acids linked together by peptide bonds. The activity and behaviour of the proteins is dependent upon the number and arrangement of amino acids in the polymer. In multicellular organisms there are substantial differences in the range of proteins found in various tissues and organs. In simple unicellular organisms such as bacteria, the proteins present vary both qualitatively and quantitatively in time and in different environments.

3.2 In spite of their importance to the living cell, proteins lack the ability to reproduce themselves and the information necessary for specifying the structure of each protein is stored in the cell in the form of sequences of nucleotide bases in the molecule deoxyribonucleic acid (DNA). Each unit of information which specifies the sequence of a particular protein is called a gene. In simple prokaryotes (organisms such as bacteria, viruses, and blue-green algae) the sequence of genes is carried on a single molecule which may be linear or circular. In contrast, in the more complex eukaryotes (all higher organisms including man), the genes are distributed amongst a number of different linear DNA molecules which are called chromosomes. Those genes which are present on the same chromosome are said to be linked. The number, shape and structure of chromosomes are specific for any given organism. In both the prokaryotes and the eukaryotes, the genes are stored as a sequence of bases in DNA. They are copied before each cell division and are passed to each of the products of that division. Thus, each cell should contain a complete set of instructions. Mutations are changes in these instructions, and may take the form of alterations within a gene and hence alterations in the protein for which it codes, or even losses of pieces of information. Once a mutation has occurred it will be perpetuated each time new copies are made.

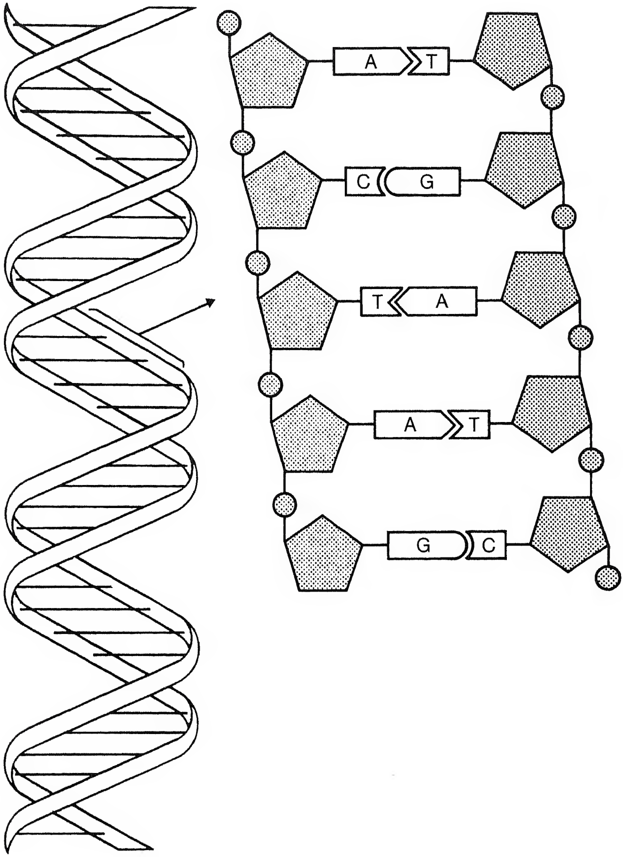
3.3 To make use of the information encoded in a particular gene it is first copied into messenger molecules (messenger RNA or mRNA) by a process called transcription. The messenger RNA is then transported to the cytoplasm where it is translated into a particular sequence of amino acids encoded by the gene. It is this amino acid sequence which will determine the shape of the protein molecule and endow it with its activity.

3.4 The following sections describe these events and the component parts of the system in sufficient detail for the reader to have a clear idea of the scientific basis of genetic toxicology. Some of the terms used might be new to the reader; a glossary has been included (Appendix 2) which explains them.

3.5 In a topological sense, the DNA molecule is something like a ladder twisted around a central axis, where the sides of the ladder are a sequence of deoxyribose sugar molecules, with the 3′ position of one sugar molecule linked to the 5′ position of its neighbour in a systematic way by phosphate molecules (see Figure 3.1). These sugar-phosphate back-bones are anti-parallel ie the 3′ OH terminus of one strand is adjacent to the 5′ phosphate of the other. Thus, the orientations of the two strands of the helix are opposite. The rungs of the ladder are made up of complementary pairs of nitrogen-containing pyrimidine and purine bases. The remarkable feature of this simple structure is that there are just two complementary pairs of bases, viz adenine (A; a purine) pairs with thymine (T; a pyrimidine), guanine (G; a purine) pairs with cytosine (C; a pyrimidine). These paired bases form a flat molecular complex within the structure of the DNA. It is the sequence of these four bases taken in groups of three along one side of the ladder that constitutes the genetic code for the amino acid sequences in the polypeptides of proteins. Base-pairing means that the two strands of the helix are complementary and thus, if one strand has the base sequence AATGCT, the other has the sequence TTACGA if read in the same direction. In this way, the replica of each strand is given by the base sequence of its complementary strand during DNA replication.

3.6 During the early studies of the structure of DNA, two forms of the molecule were observed, namely the A-form which is found at 66% relative humidity and the B-form which is found at 92% relative humidity. The A-form of DNA has 11 base pairs per turn of the helix with the planes of the base pairs tilted 20° away from the perpendicular helical axis. In the B-form, which represents the structure found in the high water-content conditions of the cell, there are 10 base pairs per turn of the helix and the base pairs are perpendicular to the helical axis. Both these structures are right-handed helices and the B-form represents the structure described by Watson and Crick (1953). In the B-form, the DNA helix displays both a deep and wide major and a shallow, narrow minor external groove in its conformational structure. Both grooves are large enough to allow some types of molecules to come into contact with the DNA bases, although atoms which lie within the major groove of the helix are reached more readily. Under some conditions, such as those of high-ionic-strengths, the DNA may assume the condition of a left-handed zig-zag structure called the Z-form. This molecule has many conformational differences from B-DNA and has an irregular backbone and only one deep conformational groove. Z-DNA is particularly favoured in regions of alternating cytosine and guanine bases and has been detected in chromosomal DNA where its formation has been implicated in the regulation of gene activity. The conformational differences between the B- and the Z-forms of the helix mean that there may be differences in the accessibility of the bases to the action of some agents in different regions of a DNA molecule.

Figure 3.1: *Helical structure of DNA*



On the left is shown a short length of the double helix. On the right is shown a short length of the DNA strand untwisted to show the positions of the sugars, phosphates and the purine and pyrimidine bases. (We are grateful to the publishers of Biology: A functional approach. 4th Edition, Roberts MBV. Nelson, Walton-on-Thames, 1986 for permission to reprint this diagram.)

3.7 The helical DNA molecule with its store of genetic information is primarily organised into either a single filamentous thread or distributed amongst a number of chromosomes. When present in the former condition, the organisms are called prokaryotes or when found in the latter condition are called eukaryotes. An elaborate mechanism has evolved in eukaryotes to ensure that all progeny cells receive a full complement of genetic material at each cell division. DNA is not confined to the chromosomes and is also found in organelles such as the mitochondria (see paragraph 3.29).

3.8 The basic unit of information in the DNA is the codon. This consists of a triplet of nucleotide bases that codes for the insertion of a specific amino acid into a polypeptide chain and the appropriate stop and start signal. As there are four different nucleotide bases in the DNA, 64 combinations are possible with a three-letter code. Polypeptides may contain combinations of about 20 different amino acids and this 64 combination genetic code provides sufficient information to code for all possible amino acids and the start and stop signals for polypeptide synthesis. Four codons are used as the signals for defining the length of a genetic message. These are the start signal TAC (or in some instances CAC) and the stop and termination signals TTA, CTA and TCA. Therefore, 60 codons are available for defining the position of the 20 amino acids and the genetic code is said to be 'redundant' or 'degenerate' ie some amino acids are coded for by more than one triplet. For example, phenylalanine is coded by the triplets AAA, GAA, TAA, CAA, whereas tryptophan is uniquely coded for by the triplet CCA except in mitochondria. The code is universal for all genetic information stored in chromosomes, TCA acting as a 'full-stop' in all species. However, in mitochondrial DNA it codes for the insertion of tryptophan. There are other examples of the genetic code for certain cell organelles differing from the universal genetic code. This universality of the genetic code in chromosomal DNA has implications for mutagenesis; if a chemical is capable of producing mutations in a simple prokaryote such as a bacterium it has the potential of producing similar types of mutations in eukaryotes including man if it is able to gain access to their DNA.

DNA polymerisation

3.9 The DNA molecules, therefore, are the repositories of the information on which the cell depends for its structure and function. Every time the cell divides, this information must be copied accurately so that each daughter cell possesses a complete inventory of instructions for making and regulating the production of its component parts. In chemical terms, this means that the replication of DNA molecules must be accomplished with great fidelity.

Determinants of fidelity

3.10 When DNA replicates, the two complementary strands separate and each serves as a template directing the polymerisation of a strand complementary to it. Polymerisation is catalysed by enzymes known as DNA polymerases and it proceeds from $5'$ to $3'$ by the successive addition of a deoxynucleotide triphosphate (dNTP) to the free 3′OH end of the growing chain. The specific pairing between adenine and thymine and between guanine and cytosine determines which dNTP will be inserted next. Thus the sequence in the template strand determines the sequence of the growing complementary strand. The accuracy of base-pairing is determined primarily by the differences in free energy between correct and incorrect base pairs. The larger this is the more accurate is replication. Measurements of free energy differences are difficult to make, but estimates that have been made suggest that error rates during DNA replication should be much higher than they are. The difference between the expected and the observed error rates is attributable in part to the DNA polymerases themselves. These enzymes can actively select the correct bases for pairing with the template and often edit insertion errors. How they select the next correct base as they proceed along the template is not known, but it has been suggested that the hydrophobic conditions at the active site of the enzyme augment free energy differences, decreasing the probability of a mispairing. Many polymerases also possess 3′–> 5′ exonuclease activity. This allows them to remove the base which has just been inserted if it is wrongly paired. It is in effect an 'editing' function which ensures the integrity of the message.

3.11 The accuracy of polymerisation when carried out in the test tube can be altered by changing the reaction conditions. DNA polymerases require the presence of Mg2+ ions for their operation. However Mg2+ can be replaced by other divalent cations but with a reduction in fidelity of replication. The mutagenic effects of Mn2+ probably stem from its effects on DNA polymerases as substitution of Mg2+ with Mn2+ increases the error rates. Biasing the dNTP pools in favour of one of the four bases increases the frequency with which this base is inserted. Several gene products also play a role in the accuracy of polymerisation, some by binding to DNA. However their effects are comparatively slight compared with the role played by the polymerases themselves.

3.12 Newly synthesised DNA may later be modified chemically. For example, methylation of the adenine residues of particular base sequences is known to occur and this is important in some organisms for directing a further 'back-up' correction system that operates immediately after polymerisation. This process, mismatch repair, recognises any mismatched bases and removes them from the newly synthesised strand so that they can be replaced by the correct bases. Mismatch repair appears to operate preferentially on undermethylated strands and thus leads to the removal of bases from the growing strand rather than the template. There is also evidence for the existence of mismatch correction systems that do not depend on methylation to identify parent strands.

The consequences of mistakes during replication

3.13 An obvious consequence of uncorrected misinsertion at replication is the substitution of one base for another. When a purine is replaced by another purine or a pyrimidine by another pyrimidine it is called a transition. The replacement of a purine by a pyrimidine and vice versa is called transversion. Such mutations may affect the functioning of the gene products, the level of the effect ranging from the undetectable to the severe. Since the genetic code is degenerate, with most amino acids being coded for by more than one triplet, a base-pair substitution may simply convert one codon for a particular amino acid to another codon for the same amino acid. Even when substitution results in a new amino acid, it may be an acceptable substitute. However, some amino acid changes can have drastic effects on the folding of the polypeptide chains or on the configuration of the active site of an enzyme. Others may have drastic effects because they convert a normally coding triplet into one of the three termination signals with the result that translation of the sequence is terminated prematurely. Such changes are usually accompanied by loss of function of the gene product.

3.14 A less obvious consequence of replicational errors is the induction of frameshift mutations. These are characterised by the addition or deletion of bases in the newly synthesised chain and, when this changes the reading frame, a severe effect is nearly always produced. Frameshift mutations appear to result from structural distortions in the template or slippage of the growing polynucleotide chain on the template, so that extra bases can be inserted or bases deleted. Mismatch repair will often correct frameshift mutations as well as base-pair substitutions. As a result of conformational changes in the template strand, more extensive changes (eg deletions) are also produced.

DNA damage and repair

DNA damage

3.15 The components of DNA (bases and the sugar-phosphate backbone) can be damaged by chemical and physical agents. The lesions produced are not mutations as such but they can lead to alterations in base sequence which are heritable changes. In most, but not all cases, DNA replication is the event which seems to be primarily involved in this step. It would be inappropriate here to provide a comprehensive description of the many types of DNA lesion known at the present time and the reader is referred to some of the more recent reviews on the subject. An important class of mutagen, the alkylating agents, can attack and form covalent adducts with the components of the DNA molecule in at least 20 different positions. Some of the less electronegative nucleophilic positions are attacked only by strongly electrophilic agents. Among the most important positions for mutagenesis are the 0^6 position of guanine and the 0^4 position of thymine, since adduct formation at these sites interferes with hydrogen bonding in the DNA helix. As mentioned earlier, the conformational shape of the DNA molecule can influence the accessibility of different sites to chemical mutagens. It is sometimes very difficult to determine which of several types of damage is significant for mutagenesis. For example, in some organisms, the cyclobutyl dimer induced by ultra violet (UV)—irradiation appears to play a central role in UV mutagenesis, but the 6–4 Py0 dimer is clearly important in other situations.

DNA repair

3.16 It is perhaps not surprising that living things have evolved a battery of enzymes to repair a variety of types of lesions before they encounter the replication complex and perturb its accuracy. Repair systems differ markedly in their specificity and precise mode of action, but many of them operate in a generally similar way. They recognise the lesion (which is usually confined to a single strand) and excise either it or the segment of the single strand containing it. They then make use of the information in the undamaged complementary strand exposed by the excision event to fill the gap with the correct bases. Repair processes of this type are generally 'error-free' in operation. Examples of this general type of repair include the damage-specific DNA glycosylases and their associated endonuclease activity and excision repair of the type seen in the bacterium *E. coli* which depends on the products of several DNA repair genes. Although many repair systems are of this general type, other important ones exist. For example, photoreactivation is a light-dependent repair system of great evolutionary importance since it monomerises or splits pyrimidine dimers induced in DNA by ultraviolet light. Alkyltransfer, another system of enzyme repair, involves the acceptance by a specific protein of the alkyl moieties from 0^6 alkylated guanine molecules in DNA. Yet another important repair function recognised in *E. coli* is post-replication-repair which depends on recombinational exchanges between daughter helices using parental sequences to replace missing information in the daughter strands synthesised on damaged templates (see below).

DNA lesions and the production of mutations

3.17 Repair is often incomplete. The remaining damage can then be encountered as template lesions by DNA polymerases. Basically two types of DNA damage exist, one of which blocks DNA replication and another which does not. However, this distinction is not clear cut. Polymerases differ in their response to lesions; damage which blocks the progress of one polymerase might not block another.

3.18 As part of the coordinated response of *E. coli* to UV treatment (the so-called SOS response), a series of gene products is induced which aids in the survival of the cell. Part of this response appears to involve gene products which permit the polymerase to by-pass the photoproducts which would normally block its passage along the DNA strand. It does this with an increased probability of error. The evidence available at present suggests that both base-pair substitution and frameshift mutations can result. Eukaryotic systems appear to be less dependent on inducible gene products in by-passing DNA lesions. However, there is evidence from studies of yeast to suggest that inducible functions exist which may be connected with error-prone events and this may also be true of other eukaryotes. Thus, viability of a mutagen-exposed cell is achieved at the expense of an increased probability of the induction of mutations.

3.19 The lesions which fail to block DNA replication may promote pairing mistakes. Even the natural bases can exist as tautomeric alternatives that have different pairing properties. If a base-analogue is incorporated into the DNA it often assumes its tautomeric form which has a greater probability of enhanced mispairing thus giving rise to mutations at each DNA replication.

Gene expression

3.20 Gene expression is the process whereby the genetic information encoded as base sequences in the DNA molecule is transcribed to messenger RNA molecules and then into a sequence of amino acids in polypeptides. These intermediary RNA molecules are synthesised during the process of transcription using the base sequence of one of the strands of the DNA helix by a polymerisation reaction catalysed by the enzymes the DNA-dependent RNA polymerases. The precursors of RNA synthesis are four ribonucleotide-5′-triphosphates, namely ATP, GTP, CTP and UTP. These precursors are polymerised into a sequence which is dependent upon the order of bases of the DNA template with the resultant RNA chain growing in a 5′ to 3′ direction. Within the template DNA there are sequences for the initiation and termination of the specific piece of information which codes for each gene product. Although an individual cell contains genes for all its functions only a fraction of this information might be required at any given time. Thus different families of messenger RNA are synthesised from different template genes at specific stages of differentiation, in different tissues or in different environments. Messenger RNA is one of the three major classes of RNA molecules which are synthesised from DNA templates, the others being ribosomal RNA and transfer RNA which have different functions in protein synthesis. Messenger RNA is the molecule responsible for transmission of the information coding for polypeptide sequences to the ribosomes which are the sites of protein synthesis. These cytoplasmic organelles are made up of three classes of stable ribosomal RNA molecules and a variety of different proteins with various functions. Transfer RNA acts as an intermediary molecule in protein synthesis by lining up the amino acids at the ribosome in the order determined by the messenger RNA sequence. Approximately 50 different stable transfer RNA molecules carry a three-nucleotide anti-codon which recognises the codon of the messenger molecule which codes for each specific amino acid. The polypeptides are synthesised in a sequence of amino acids which corresponds to the order of the triplets of bases of this messenger RNA molecule. In prokaryotes, the molecule which reaches the ribosome is essentially the same as that which was synthesised on the DNA template and has only a short life time in the cells (as little as 30 seconds) before being broken down. However, in the eukaryotic cell, the DNA may also contain both non-coding regions, the introns, and coding regions, the exons. Thus the messenger RNA synthesised can contain regions which interrupt the sequence of the message of each particular gene. Introns are removed from the messenger RNA molecule by a number of events collectively described as RNA-processing and the resulting processed messenger RNA molecule may have a long life-span in the cell. The presence of introns in the DNA of eukaryotes means that there

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are regions of this DNA whose modification by mutagenic agents might not interfere with the integrity of the gene.

3.21 The DNA segment which corresponds to a single polypeptide chain is called a cistron. In eukaryotes, each messenger RNA molecule encodes for a single polypeptide chain and is said to be monocistronic. However, in prokaryotes the messenger RNA may encode the information for several polypeptide chains and is therefore polycistronic. These differences in the informational content of messenger RNA molecules reflect the different modes of organisation of cistrons in prokaryotic and eukaryotic chromosomes and their respective mechanisms for regulating gene function.

The organisation of the genetic material—genes and chromosomes

3.22 The basic unit of the genetic material is the gene. A gene is a sequence of bases in a DNA molecule which encodes the information for a functional unit of inheritance, eg a polypeptide for a structural gene or enzyme or a base sequence of a controlling or regulatory gene. In all organisms the DNA is present in the cell as one or more giant filamentous molecules consisting of sequences of genes interspersed in a species-specific way with non-coding repetitive sequences of bases (introns), some of which in higher eukaryotes are relatively enormous. Such an arrangement of genes together with associated protein and enzymic systems makes up the chromosome. The composition, structure and cellular location of chromosomes differs greatly between cells of prokaryotes and eukaryotes. In prokaryotes, the chromosomes basically consist of the DNA of the gene string, which in *E. coli*, for example, is a single circular macromolecule of double-stranded DNA comprising about four million base pairs. This 'chromosome' is organised with various proteins and other substances into a discrete 'nuclear body' with a regular structure. In eukaryotes, the chromosomes, usually many per cell, are more complex structures containing a variety of proteins, enzyme systems and RNA in addition to DNA. Whereas in prokaryotes the nuclear body is free in the cytoplasm, in eukaryotes the chromosomes are enclosed within the membrane of the nucleus except at cell division ie at meiosis in germ cells and mitosis in somatic cells.

3.23 All prokaryotes and some lower eukaryotes, eg certain fungi, are haploid, ie their cells contain only one set of genes, usually arranged as a single gene sequence, or, if the genes are distributed into separate chromosomes, each chromosome consists of a different set of genes. A complete set of genes is called a genome and embodies the sum total of the genetic information contained in the individual (ie unpaired) genes in an organism.

Organisation of the genetic material—the genome, ploidy, dominance, recessivity

3.24 The organisation of coding sequence of DNA has been summarised earlier. An average-sized prokaryotic polypeptide of 200–300 amino acids will require the coding information provided by three times as many nucleotide bases plus start and stop codons and the upstream and downstream flanking regions needed for processing the message. Eukaryotic genes may be much larger. Even without introns a coding sequence length of 2500–3000 bases is not uncommon. When introns are present the total sequence of introns and exons can be tens of thousands of bases in length. In prokaryotes, groups of genes with related functions are often close to each other spatially and are regulated as a unit so that each produces its product in concert with the others. However this need not be so. The genes of the SOS network in *E. coli* are all regulated by the product of the lexA gene, but they are scattered throughout the genetic map of the organism. In eukaryotes, the clustering of genes involved in a single metabolic pathway is far less common.

3.25 Some lower and all higher eukaryotes are diploid, ie their somatic cells contain two copies of each chromosome. These matching pairs are called homologues. In sexually reproducing diploid species, one of the homologous chromosomes is derived from the father and the other from the mother. Each gene occupies a precise position or locus along the length of the DNA molecule of a given chromosome. Thus, with one important exception to be mentioned below, homologous chromosomes are functionally and spatially matched gene for gene along their entire length, even though one gene of the pair comes from the mother and the other from the father. Maternal and paternal genes may be the same or they may differ. Different functional forms of the same gene are known as alleles, and it is important to note that they do not necessarily perform their function in the same way. For example, one allele of a gene may produce the normal type and amount of enzyme, while the other may produce less enzyme, or a temperature sensitive enzyme or none at all. Diploid organisms are said to be homozygous for a given allele at a given locus when they carry identical versions of this allele, and heterozygous when they carry different versions. Studies in mice have indicated that maternally- and paternally- derived loci or regions of chromosomes might not function equally during embryogenesis or fetal development. The phenomenon might not be restricted to this species.

3.26 Heterozygosity leads to complications in gene expression. Sometimes two different alleles express themselves independently of each other; this is called co-dominance, and is seen, for example, in the A and B pair of alleles of the human ABO blood group. Often, one of two alleles dominates development and function to the full or partial exclusion of the effects of the other allele. In these cases, the two alleles are respectively called dominant (or partially dominant) and recessive (or partially recessive). An example of a recessive (or partially recessive) allele in man is the allele for phenylketonuria. The definition of dominance is somewhat ambiguous. A fully dominant allele of a gene should be equally well expressed in the heterozygous or in the homozygous states. This is true for many normal alleles in relation to fully recessive mutant ones (eg alleles for certain kinds of deafness). In man, however, dominant mutant genes are often known only in heterozygotes because they are so rare that homozygotes have never been seen, because the heterozygotes do not produce offspring, or because the homozygotes die as early abortions. In these cases, especially when dealing with human abnormalities, a mutant allele is said to be dominant when it manifests itself in the heterozygote although knowledge of the homozygote would probably show that it is only partially dominant. An example of a condition caused by a dominant allele in man is brachydactyly.

3.27 The one exception of the rule that all genes in mammals are paired is that for X-linked genes, ie genes on the X chromosomes, of which a male has only one copy and a female two copies. Its partner in the male, the *Y* chromosome, has been shown in man to have specific DNA sequences in common with the X, these being located in a very short region that pairs with a region of the X at meiosis, (see later); but only in the mouse is there any evidence of a gene carried by both chromosomes that is actually transcribed. The general rule, however, is that X-borne genes, whether dominant or recessive, do not have a counterpart on the Y and will manifest themselves as sex-linked (or X-linked) characteristics or diseases. The situation in females is complicated by the fact that, with the exception of genes that lie in or close to the X-Y pairing region, only one of the two X chromosomes expresses its genes in any given cell of somatic tissue and this may be either paternally or maternally derived.

3.28 Although most of the genetic material of cells is contained within the chromosomes and, in the case of the eukaryotes, is contained within a nuclear membrane, DNA may also be found at other sites within the cell. In bacteria, up to 8% of the total genetic material may be found in the form of separate circles of DNA (or in some cases as RNA) called plasmids. These molecules of DNA can be stably inherited without being linked to the chromosome and in some cases may be transferred from cell to cell independently of the chromosome. Plasmids may carry a variety of genes which code for various toxins and for resistance to a range of antibiotics. In some cases, the bacterial plasmids may carry specific repair genes whose activity has been exploited in the construction of specific mutagenicity tester strains. For example, the plasmid pKM101 carries the genes *mucC,D*$^+$, which confer increased mutability on some strains of bacteria. There are also examples of plasmids which have been constructed so that they carry copies of genes normally found on the prokaryotic chromosome. The plasmid PAQ1, which carries copies of the histidine G gene which is normally found in the chromosome of *Salmonella typhimurium* is an example.

3.29 In eukaryotes, extranuclear DNA is found in a variety of organelles including the mitochondria and chloroplasts of green plants. The activity of extranuclear genes is most well understood in organisms such as yeast where extensive genetic maps have been constructed of this extra-nuclear genome. Mitochondrial DNA represents a potential target for mutagenic agents additional to chromosomal DNA. In yeast, mutation studies may be performed using both the chromosomal and mitochondrial genome. The complete sequence of human mitochondrial DNA (consisting of 16,569 base pairs) has been determined. This sequence contains information specifying ribosomal subunits, transfer RNA molecules and subunits of the enzyme cytochrome oxidase.

The transmission of genetic information from one generation of cells or organisms to the next in eukaryotes

3.30 During growth, the body cells (or 'somatic' cells) divide by a process called mitosis. This involves cell division following DNA replication and is concerned with the production of new generations of somatic cells within an individual. However, early in the development of an embryo, a group of cells, the germ cells, are set aside to be the precursors of the future gametes (ova or spermatozoa). Germ cell development, gametogenesis, is quite distinct from the development of somatic tissues. A mature gamete possesses a haploid set of chromosomes; the production of haploid gametes from diploid germ cells is called meiosis. The fusion of the female haploid gamete with the male haploid gamete produces the diploid zygote, which, by subsequent mitotic cell divisions, produces a new individual of the next generation.

3.31 During transcription into messenger RNA the nuclear chromosomal material (chromatin) in a eukaryotic cell is dispersed in its filamentous form throughout the nucleus and is not visible by conventional optical microscopical techniques. Prior to mitosis, the production of messenger RNA ceases and DNA replication takes place. In the early stages of mitosis, prophase, the chromatin complex condenses and forms visible chromosomes. Each chromosome at this stage consists of a pair of identical double helices of DNA compacted and condensed to form a pair of distinct chromatids linked together at a point of primary constriction called the centromere which is associated with a protein structure known as the kinetochore (spindle attachment). Disappearance of the nuclear membrane marks the end of prophase, the beginning of the organisation of the spindle and the transition into the next stage, metaphase. At metaphase the contracted chromosomes move to the cell equator under the control of spindle microtubules. Spindle fibres attach to the centromeres/ kinetochore of each chromatid and, at anaphase, the centromere of each pair of chromatids splits and the individual chromatids separate and are moved to opposite poles of the cell as new chromosomes. In the final phase, the spindle breaks down, a partition is developed in the equatorial region which divides the cell into two halves, the chromosomes become diffused, and two new nuclei develop.

3.32 The drug colchicine inhibits the formation of the spindle at metaphase and thus can be used to prevent the organisation of the chromosomes to the metaphase equatorial region. The chromosomes remain widely separated, become contracted and individually more recognisable. It was by the use of techniques such as this that the characteristic number and shape of the chromosomal pattern known as the karyotype for cells of different mammals was established. Man has 46 chromosomes ($2n = 46$), consisting of 22 pairs of homologous chromosomes, called autosomes, and two sex chromosomes, X and Y in the male, or XX in the female. The chromosomes can be assigned into groups according to their size and the location of their centromeres. Other species have chromosomes of different size, shape and number and of course different gene complements. Using a number of techniques, chromosomes may be seen to have characteristic banding patterns along their length. These banding patterns are specific for each individual chromosome.

3.33 Meiosis differs from mitosis in several important ways. At the beginning of meiosis, homologous chromosomes pair with one another and, having exchanged genetic material by a process of crossing-over, separate from each other in the first meiotic division. Thus, in contrast to mitosis, the chromosome number of the daughter cells is half that of the mother cell and each chromosome still consists of two chromatids. Because of the exchange of genetic material in the first meiotic division, the two chromatids of a chromosome are usually no longer identical. The second meiotic division resembles a mitotic one in that it splits the centromeres, separates the chromatids and does not change the number of chromosomes. Thus, in meiosis, each diploid germ cell has the potential to produce four haploid cells. This occurs during spermatogenesis in the male and the four products of each meiotic division mature to form four spermatozoa. In the female, only one product of the first division continues development, the other forming a polar body. Similarly, at the second division only one product is retained, the other forming the second polar body. Overall, therefore, only one ovum and two polar bodies result.

3.34 Thus far, the basic elements of the hereditary processes have been outlined, from DNA replication and transcription to somatic and germ-cell division. By these mechanisms, a complete set of genes is transferred, either to the next generation of somatic cells or (after fertilisation) to future individuals. However, these complex processes can go wrong, with the generation of heritable mistakes or mutations, so that a functionally changed and often imperfect set of instructions is transmitted, resulting in defective cells or individuals. Mutagenesis is the name of this process of generating mutations. It can occur spontaneously or be induced by chemical or physical agents, which are then described as 'mutagenic'. The detection of chemicals that might be human mutagens is a rapidly expanding field that runs parallel with a similar expansion of knowledge of the molecular biology of the genetic processes themselves. An added stimulus for this interest is that, apart from the serious implications for future generations by the addition of further genetic or partly genetic diseases to the already far-from-negligible current burden, there is strong evidence that mutations in somatic cells can lead to the production of neoplasia and cancer (see Chapter 4). Before proceeding to more general considerations of mutagenesis there are some imporant aspects of meiosis that have considerable significance from the view-point of mutagenesis at the chromosomal level.

Genetic variability: independent assortment, crossing-over

3.35 Although all four products of meiosis contain a complete set of chromosomes, they are far from being genetically identical. Two processes are responsible for creating genetic variability at meiosis. They form the basis for some of the suggested methods of assessing mutagenic hazard and hence need further consideration.

Independent assortment

3.36 The first mechanism, called independent assortment by the early geneticists, rests on the fact that each pair of homologous chromosomes segregates independently of all the others. Thus, the daughter cells receive different combinations of maternally and paternally derived chromosomes. If the maternally derived chromosomes are indicated by the suffix m, and the paternally derived ones by the suffix p, a sexually produced individual has the chromosome set 1m1p; 2m2p etc. For any given chromosome pair, a gamete has an equal chance of containing either an m or a p chromosome. Thus for two chromosomes there are four possible combinations ie 1m2m 1p2p 1p2m 1m2p. It can be seen that, for the 23 chromosome pairs of man, the variability created in this way is great. It is, however, not the only source of variability.

Meiotic recombination and crossing-over

3.37 A second mechanism, called crossing-over, results in a mixture of paternal and maternal genes within the same chromosome ie between linked genes. At the beginning of meiosis, when homologous chromosomes are closely paired, they exchange sections with each other in a precise manner. Pairing and crossing-over may be necessary for the orderly segregation of homologous chromosomes. Each single cross-over takes place between two of the four chromatids, and successive exchanges (the number of which varies with the length of the chromosomes) may involve different chromatids. The detailed mechanism of exchange is not known, but it is possible that processes similar to DNA repair are involved. The process, known as recombination, is under strict genetic control and even though the molecular details of the process are not fully worked out, there are important cytological and genetical consequences. The cytological results can be observed just before first meiotic metaphase when previously paired homologous chromosomes begin to separate from each other. At this stage, joins can be seen between two of the four chromatids so that some of the paired chromosomes take on the appearance of crosses. These joins are called chiasmata and each chiasma (= a cross) corresponds to a crossing-over. The genetical result, and indeed the fundamental outcome, of recombination is the reshuffling of alleles within the chromosomes which occurs at chromatid level. In this way, individual chromatids may emerge from the meiotic process with different combinations of alleles. Thus a recombinant chromatid whose centromeric region, for example, is of paternal derivation, may carry in sequence next to it a stretch of DNA of maternal origin, and this in turn, may be followed by a further stretch of the paternal homologue. This ensures that no two individuals, barring identical twins produced by the splitting of a single zygote, are genetically alike. Recombination is responsible for the vast amount of diversity in human populations.

Mitotic recombination

3.38 Crossing-over can also occur during mitotic division when pairing occasionally happens between individual chromosomes. These mitotic crossing-over events are most readily detected and quantified in fungi. The recombination events between genes on a chromosome are reciprocal in nature. However non-reciprocal events can also occur during genetic exchanges within a gene by a process called gene conversion.

3.39 Gene conversion can take place at meiosis or mitosis. Gene conversion at mitosis in yeast has been widely used in testing for genetic activity. There appears to be a good correlation between mutagenic activity and the induction of gene conversion, regardless of the type of mutagen being considered. The process takes place during the interaction between two homologous DNA duplexes and is often associated with reciprocal recombination between genes on either side of the region being studied. During gene conversion, one strand of one of the duplexes exchanges positions with an equivalent segment of a strand of the same polarity in the other duplex. If the two duplexes are identical, the newly associated strands will pair perfectly. If, however, the original duplexes differ in a single base pair, the newly associated strands will contain mismatches at that point. Mismatch repair now recognises the mismatch and corrects it either in favour of the wild-type or the mutant base sequence. Thus, what was originally a mutant site can be converted precisely back into the original wild-type site. This is the basis of the yeast mitotic gene conversion test. The two original duplexes differ in two well-separated places within the same gene. One has one mutant allele, the other has the second mutant allele. During gene conversion either mutant can be converted to be wild-type form and when this happens a duplex is generated without either mutation. Mutagenic agents probably facilitate this process because they break the DNA directly or indirectly and enable strand exchanges to occur more readily. They may also induce enzyme systems involved in recombination. The induction of mitotic gene conversion may thus be used as an indirect test for mutagenicity.

Organisation of genes on chromosomes

3.40 Gene-mapping is an important part of genetical research. Until the late 1970's, this depended on the use of recombination data obtained from crosses between genetically different individuals. When cells undergo meiotic division, genes may be exchanged between homologous chromosomes. The closer together the genes are linked, the less is the likelihood of recombination occurring between them. The numerical values used to construct genetic maps were essentially probabilities for exchanges of information within defined chromosomal intervals between linked genes. The maps produced were thus not accurate representations of the physical distances between genes. Also, because access to as many progeny as possible is desirable in map construction, the most detailed maps were obtained using prokaryotes and lower eukaryotes. Human gene-mapping was slow and depended mainly on the analysis of family lineage data for sex-linked genes. With the advent of techniques for making human/rodent cell hybrids, parasexual methods of analysis could be used to determine the chromosomal assignment of many human genes. When the use of translocation and deletion mapping was included and molecular methods for the unambiguous identification of specific DNA sequences (eg by DNA/DNA and RNA/DNA hybridisation) became available, the physical mapping of genes in man in particular accelerated rapidly. More recently, the technique of 'tagging' particular alleles by means of restriction-site alterations has simplified the process of prenatal diagnosis and the identification of 'at risk' individuals. For further information on these techniques the reader is referred to the bibliography (see Appendix 1).

Sister chromatid exchanges

3.41 Exchanges in which genetic markers are recombined may take place between homologous chromosomes at both meiosis and mitosis. Exchanges between sister chromatids, sister chromatid exchange (SCE), are difficult to demonstrate by purely genetic techniques since they involve the normally identical replication products of a single chromosome. However, if the chromosomes are made to replicate twice in the presence of an analogue of the base uridine called 5-bromodeoxyuridine (5BUdR) one of the final replication products in a pair of sister chromatids will carry the analog in both strands of its DNA helix, but the other will have only one analog-containing strand in its DNA helix. The two chromatids will then react differently with a fluorescent dye and can be distinguished under the microscope. If exchanges between the two sisters have taken place they appear as alternate strongly and weakly fluorescing or staining segments along the length of the bivalent. The mechanism of sister chromatid exchange is still unknown, but as with reciprocal recombination and gene conversion, the frequency with which it occurs can be enhanced by mutagenic agents. Thus it can serve as an indicator of genetic damage although of itself it usually has no genetic consequences. SCE appears to be more frequent at mitosis than at meiosis.

3.42 The precise exchanges which take place between homologous chromosomes during crossing-over involve breaks in the DNA backbone, but they should not be equated with the breaks induced by chemicals and radiation which can generate rearrangements between non-homologous chromosome segments. As noted for gene conversion (see paragraph 3.39), augmentation of recombination can result from the introduction of single strand breaks into the DNA, but it is possible that the synthesis of the enzymes involved in strand exchange may also be enhanced. It is noteworthy that the chromosome-breaking ability ('clastogenicity') of a compound is not always a good indicator of its ability to induce SCE.

3.43 One of the steps in the process of crossing-over is breakage of single strands of DNA. This is probably the reason why many mutagens increase the frequency of genetic recombination or induce it in cells in which it does not usually occur, eg male germ cells of the fruit-fly *Drosophila*. While meiotic recombination *per se* does not usually create a genetic risk, a substance that induces it is also likely to produce other types of chromosome damage that do constitute a hazard. These are the breakages of chromatids or chromosomes which are often followed by the formation of new 'rearrangements' between the fragments (see paragraph 3.38). Since it is much easier to score recombination than chromosome-breakage and -rearrangement in organisms such as fungi, the ability of a substance to produce the former is often taken as an indicator of its ability to produce the latter. It should however be understood that crossing-over differs from the formation of chromosome rearrangements in several essential features. Rearrangements can be formed at random from any fragments that are close enough for rejoining; crossing-over occurs only between homologous chromosomes, with the points of breakage being identical in both. Rearrangements are formed without regard to the orientation of the chromosomal segment, hence the production of inversions of gene sequences. In crossing-over, the original orientation of the segment to the centromere is almost always preserved; thus the genes in the new strand are arranged in exactly the same order as they were before crossing-over. However, instead of the normal X-type exchange, it is possible to have a U-type cross-over which obviously does not maintain the normal gene sequence; this type of exchange occurs regularly, although at very low frequency, and can be identified by the formation of bridges and fragments at anaphase I. Although chromosome-breakage and subsequent rearrangement and crossing-over can have one or more chemical steps in common, they clearly are not identical processes and caution has to be used in interpreting results of cross-over tests in terms of mutagenic potentialities. The same caution needs to be exercised in interpreting the sister chromatid exchanges (SCE) which have been used for mutagen testing. These are due to exchanges, not between homologous chromosomes, but between segments of sister chromatids of the same chromosome at mitosis. There are correlations between the ability of a treatment to produce SCE and chromosome rearrangements, but again there are also differences.

Mutations and their induction

3.44 Knowledge of how changes in the DNA/chromatin system can be brought about has been growing for over 50 years as a result of extensive experiments with ionising radiations and a variety of chemicals. It was the realisation that chemicals could produce DNA damage that initiated the present concern about the mutagenic hazards posed by many chemicals in common use. The growth of the study of mutagenesis and its correlation with carcinogenesis has been so great that the literature is now enormous.

3.45 When assessing the risks from chemical mutagens it is necesary to study quantitative aspects of their action, for example, the relationship between the dose and the mutational response. However, it is also necessary to investigate the metabolism of a mutagen so as to determine the extent to which it and its active metabolites are likely to reach the genetic material of somatic and germ-line cells, and what the effects in man might be. Only by a combination of mutational and pharmacokinetic studies is it possible to estimate likely genetic hazards at low exposure levels.

3.46 Broadly speaking, genetic defects in eukaryotes stem from three categories of mutational events, which are (i) at the gene level, (ii) at the individual chromosomal level and (iii) at the level of the chromosomal set, ie a change in chromosomal number.

Mutations at the gene level

3.47 Dominant and recessive point mutations may lead to a number of dominant and recessive diseases. Small deletions that are undetectable at the cytological level are believed to be the basis of many induced mutations, and rearrangements that have breakpoints in or near genes can prevent their transcription. A distinction also has to be made between the effects of dominant and recessive mutations. Dominant mutations affect any individual who has received the mutant genes from either the spermatozoon or the ovum. They therefore typically become manifest in the first generation, although those with poor penetrance could be expressed for the first time in later generations. Recessive mutations affect only those individuals that have received the mutant gene from both ovum and spermatozoon. Such mutations can be carried in heterozygotes for many generations before a homozygous child who manifests the effects of the mutation is produced. However, in view of the large burden of existing recessive genes they should not be neglected in the calculations of genetic risk. In addition, it has been shown in *Drosophila*, mice and man that many recessive mutations have small but definite effects on survival in the heterozygous state. Thus recessive mutations are likely to contribute an unknown but important component to the general genetic deterioration expected from an increased mutation frequency.

3.48 Our knowledge of mutational mechanisms is still heavily dependent on information obtained with *Escherichia coli* but the mechanisms of mutagenesis in eukaryotes may well prove to differ in their details from *E coli*. They may, for example, be less dependent on damage-inducible processes than *E coli*. However, judging from what is known of mutation in yeast, the control of induced and spontaneous error rates probably involves the products of many genes. Another factor of importance may well be the structural components of eukaryotic chromosomes. Although the *E coli* chromosome is not as 'naked' as might be believed, it seems likely that regional differences in mutagen sensitivity in eukaryotic chromosomes stem at least in part from the associated histones and other proteins. These are important factors to bear in mind when quantitative comparisons are attempted between the mutagenic responses of evolutionarily disparate groups. In qualitative terms, however, it seems likely that, where point (that is intra-genic) mutations are concerned, base pair substitutions will be promoted by similar lesions in pro- and eukaryotes, and their effects will be comparable. Evidence is now accumulating to suggest that similarities also exist between the induction of frameshift mutations in pro- and eukaryotes although the role of the mutagens in this type of event still remains obscure. At present, only limited evidence is available concerning the processes which result in the deletion of extended tracts of bases within the genes of eukaryotes. So far, however, there is no reason to doubt the predictivity of prokaryotic systems for point mutations but this may be revised as our understanding of mutagenic mechanisms in eukaryotes improves.

3.49 Even with cultured mammalian cells, mutation systems are only now becoming well enough characterised to allow fundamental studies to begin.

This raises the question of the extent to which results obtained with bacteria can be extrapolated to higher organisms, in particular to man. There are many major differences between the genetic systems of pro- and eukaryotes as discussed earlier. Mammalian cells contain a considerable amount of DNA, which consists of a specific base sequence repeated many thousands or even millions of times, which is not transcribed. However, the essential features of gene replication and the ultimate chemical interactions between mutagens and DNA are the same in all organisms, including bacteria. Thus there is reason to believe that there is a fair probability that tests for the production of gene mutations in bacteria will be of predictive value for man. This is not true, however, for chromosome breaks, which in prokaryotes are usually lethal and do not lead to the genetic consequences to be described later for eukaryotes.

Damage at the level of individual chromosomes

3.50 Primary damage to individual chromosomes consists of breakage of chromatids which must result from a discontinuity of both strands of the DNA in a chromatid. How chemical mutagens produce chromosome breakage is not yet well understood, but various lesions in DNA which are not in themselves discontinuities, will result in breakage of a chromatid as a consequence of their interference with the normal process of DNA replication. While in prokaryotes and haploid micro-organisms chromosome breaks are usually lethal this is not so for diploid eukaryotes. In these organisms, chromosome breaks may undergo three different fates, with quite different consequences for the cell:

(i) They restitute in the old order, presumably as a result of an enzymic repair mechanism. In this case no obvious cytological damage ensues.

(ii) They remain unrejoined as fragments. This may result in death of the cell at the next or one of the following mitoses. If unrejoined fragments are introduced into the zygote via a treated germ cell, the embryo may die at a very early stage from a so-called ‘dominant lethal’ mutation. While there might be other causes of dominant lethality, its production by a chemical is a fairly good indication that this substance produced chromosome breaks.

(iii) They rejoin in a new order different from the original one, resulting in chromosome rearrangements. The most important of these and their genetic consequences are:

(a) Reciprocal translocations involving the exchange of chromosomal segments between two chromosomes. Depending on the position of the centromeres in the rearranged chromosomes, this will lead to two quite different consequences. First, is the case of an asymmetrical exchange, when one of the rearranged chromosomes carries both centromeres (dicentric) while the other carries none (acentric). The zygote carrying this anomaly will usually die. Death in this situation is due to the genetic deficiency caused by the loss of the acentric fragment at cell division and also to the segregation difficulties of the dicentric. Thus this type of translocation contributes to dominant lethality.

The second case occurs with symmetrical exchanges when, on the contrary, each rearranged chromosome carries just one centromere, allowing the zygote to develop into an apparently normal individual. When, however, such heterozygotes for reciprocal translocations form germ cells at meiosis, about half their gametes will be genetically unbalanced, having duplications and deficiencies of chromosomal material. Most of the unbalanced gametes survive and lead to the production of unbalanced zygotes. The usual consequences are death shortly before or after birth, or congenital malformation in survivors.

(b) Robertsonian translocations (centric fusions) are particularly noteworthy because they involve the joining together of two chromosomes, each of which has a centromere at, or very near, one end, to produce a single metacentric or submetacentric chromosome, that is, a chromosome with its centromere at or near the middle. The precise means by which Robertsonian translocations occur is not clear, but several mechanisms are possible. When they are produced *de novo* in a germ cell and derive from breakage and rejoining in the short arms of the two chromosomes, a genetic deficiency can result as a consequence of loss of the derived acentric fragments. Many Robertsonian translocations are compatible with viability; in fact they have played an important part in evolution. However, in some instances, they pose a major genetic risk. In heterozygotes, the two arms of the translocation chromosome may pair with the two separate homologous chromosomes at meiosis, but segregation is disorderly. A proportion of the resultant germ cells either lack (nullisomy) or carry two copies (disomy) of one or other of the two chromosomes involved. As a consequence, both monosomic and trisomic embryos are produced. The former carry only one copy of a chromosome and, typically, die early. Trisomic embryos carry three copies of a chromosome and can survive to birth or beyond, according to the chromosome concerned (see paragraph 3.53). For example, if chromosome 21 is involved in the translocation, Down syndrome could result. Translocation trisomy, as this condition is called, has the added danger that, unlike non-disjunctional trisomy (see paragraph 3.52), it can be transmitted by apparently normal persons who carry the Robertsonian translocation in a balanced karyotype. Inherited Down syndrome is an example of this.

(c) Inversions result when two breaks occur in the same chromosome, the segment between them is detached, and then reinserted in its original position but the other way round. That is, the order of genes in the segment is reversed. This does not necessarily constitute a genetic hazard but, depending upon the position and size of the inversion, unbalanced gametes could result causing subsequent fetal death or congenital malformations.

(d) Deletions and deficiencies arise when two breaks occur close together in the same chromosome, the fragment between the breaks becomes detached, and the two ends of the chromosome join. The now unattached piece of the chromosome is likely to be lost at the next cell division. Very large deletions are believed to contribute to dominant lethality. Small deletions involving one or several contiguous genes are often difficult to distinguish from changes within a gene, ie point mutations. They are probably the most important genetic risk apart from non-disjunction. Just as with some recessive mutations, deletions may uncover the deleterious effect of a pre-existing recessive gene. More important is the fact that even a small string of contiguous genes is likely to contain at least one gene that is essential for survival and is probably partially dominant in heterozygotes; deletion of such a gene would act as a lethal in homozygotes.

3.51 Chemicals differ widely in the kinds of damage they produce in chromosomes and in the proportion in which they may produce the effects (a) to (d) mentioned above.

Changes at the level of the chromosome set

3.52 The maintenance of the chromosome complement or karyotype of an eukaryotic cell during somatic and germ cell division is dependent upon the accuracy of chromosome replication and the segregation of the correct numbers of chromosomes to the progeny cells. The segregation of chromosomes is dependent upon the functioning of a number of cell organelles and by a range of division-specific metabolic activities such as the synthesis and functioning of the proteins of the spindle apparatus and the attachment and movement of the chromosomes on the spindle. Occasionally, the mechanisms by which homologous chromosomes segregate into different daughter cells go wrong and progeny cells receive an incorrect number of chromosomes. Cells may thus be produced which contain abnormal chromosome numbers, whether a multiple of the complete karyotype (polyploidy) or with deviations from the normal number of individual chromosomes (aneuploidy). When both copies of a specific chromosome move into a single progeny cell and the other cell receives no copies, this event is called non-disjunction. If this occurs during mitotic cell division, the progeny cells contain one less or one more chromosome that the normal karyotype. Such cells are called monosomic and trisomic. If non-disjunction occurs during meiosis, as indicated for Robertsonian translocation-based non-disjunction (see paragraph 3.50), the subsequent normally haploid products consist of monosomic products and disomic products which on fertilisation will produce zygotes which are monosomic or trisomic for the chromosome in question.

3.53 In humans, aneuploidy in both abortuses and live births represents a significant economic and social problem which stems from the generation of abnormal aneuploid gametes during germ-cell meiosis. Aneuploidy is the most common cause of abnormalities in humans. Trisomy or monosomy of the larger chromosomes leads to early death of the embryo. However, trisomy of the smaller chromosomes can be compatible with survival, albeit with abnormal features. Examples of such trisomy are Down (trisomy 21), Edward (trisomy 18), and Patau (trisomy 13) syndromes, all of which are detrimental to the health of the affected person. The only monosomy found amongst live births is sex chromosome monosomy (XO) which is known as Turner syndrome.

3.54 Aneuploidy in somatic cells has also been implicated as playing a role in the formation and progression of human tumours. The vast majority of human tumours contain aneuploid cells, and up to 10% of tumours are monosomic or trisomic for a specific chromosome as the sole observable cytogenetic change. Amongst such tumours, trisomy 8, 9, 12 and 21 and monosomy for chromosomes 7, 22 and Y are most common. Some neoplastic conditions such as chronic lymphocytic leukemia show a high correlation (30%) with the gain of a single chromosome (in this case trisomy for chromosome 12).

3.55 Unlike the induction of point mutations and chromosome aberrations, where the predominant target of genotoxic chemicals is clearly defined as the DNA of the chromosomes, aneuploidy can result from damage to a variety of cellular targets. These include targets common to both mitotic and meiotic cell division such as the polypeptides of the nuclear spindle and its synthetic apparatus, the kinetochores which attach the chromosomes to the spindle and the centrioles which are responsible for the polar orientation of the division apparatus. In addition to these targets common to both types of divisions, there are also events that are specific to the meiotic cell, such as homologous chromosome pairing with the formation of the synaptonemal complex and the process of crossing-over. Interaction with all these 'targets' may have a potential role in the induction of aneuploidy, and interference with their functioning may lead to the production of cells with abnormal chromosome numbers.

4. Implications for carcinogenesis

The role of genetic changes in carcinogenesis

4.1 It has been appreciated for many years that genetic changes are of central importance in the development of tumours. In 1914, Boveri noted the prevalance of chromosomal abnormalities in cells from a variety of human and experimental tumours and suggested a genetic basis for neoplasia. The intervening years have seen the emergence and disappearance of a number of alternative theories, but that linking mutational changes in DNA to carcinogenesis is more strongly supported by the available evidence now than ever before.

4.2 One of the strongest indications that changes in genetic material are important in tumour development is the existence of a series of dominantly inherited conditions which predispose to cancer in man. These include familial adenomatous polyposis coli, which leads to the formation of multiple colorectal adenocarcinomas and neurofibromatosis (von Recklinghausen's disease) which is associated with an increased risk of developing malignant nerve sheath tumours and also benign and malignant tumours at some other sites. Additional evidence is provided by recent investigations into the inherited forms of retinoblastoma showing that affected individuals inherit a copy of the predisposing gene from one parent; tumours arise when a somatic mutational event takes place in the other allele of the same gene in the developing retinal cells. It seems likely that the new techniques in molecular biology will identify the genetic defect in an increasing number of human tumours in the future.

4.3 Genetic alterations may of course be induced in bacterial or mammalian cells by treatment with chemical mutagens. There is a good evidence for a very close (although not complete) positive correlation between *in vivo* mutagenicity and carcinogenicity. Many of the compounds shown to be mutagenic in whole animals have not been tested for carcinogenicity, but of those that have, the vast majority are carcinogenic. A very small number of chemicals which can convincingly be shown to induce mutations in mammals are nevertheless not detectably carcinogenic in such tests. The reasons for this are not fully understood, but it could be that conditions of the assay, such as species, dose or duration of study, might not have been capable of detecting the carcinogenic effects. Alternatively, pharmacokinetic and metabolic factors may act to prevent the mutagenic activity being expressed as tumour formation. Because of these uncertainties it seems reasonable to regard compounds which are known mutagens *in vivo* as having carcinogenic potential, even though this potential might not be demonstrable in formal carcinogenicity tests.

The role of genotoxic and non-genotoxic chemicals in carcinogenesis

4.4 The term 'genotoxic' is used here in its narrow sense to denote compounds which directly interact with DNA to cause genetic alterations in bacterial or mammalian cells. Many of these compounds act by forming adducts with specific nucleotides in the DNA. If these are not recognised and removed by the cell's repair machinery, a point mutation can result. It is relatively easy to see how such genotoxic chemicals can act as carcinogens. It is however much more difficult to envisage mechanisms by which non-genotoxic chemicals exert their effects. The term 'non-genotoxic' is used to describe substances which do not detectably interact with DNA either directly or after metabolic activation. Some of these nevertheless are carcinogens in animals, but their mode of action remains unclear. Among the possibilities which have been considered are the following:

(a) In spite of being negative in mutagenicity assays, some compounds might induce non-specific DNA damage indirectly, through the intermediary of highly reactive free radical species such as oxygen.

(b) Certain substances may act by stimulating free radical proliferation, consequently increasing the probability of a somatic mutation occurring which will enhance tumour development.

(c) It is possible that some apparently non-genotoxic carcinogens do not induce tumours directly, but act by 'promoting' the growth of cells which have undergone rare spontaneous mutations (initiated cells) which would normally remain dormant within the tissue. It is well established that initiated cells can exist in a dormant form for extended periods, only giving rise to tumours on exposure to a promoting stimulus.

(d) Another class of non-genotoxic carcinogen is represented by certain immunosuppressant drugs. Tumours can develop in patients or animals treated with these compounds as a consequence of reduced resistance to virus infection, but the alternative possibility of a defective immune surveillance against tumour cells cannot be excluded.

4.5 It should be noted that the classification of carcinogens as genotoxic or non-genotoxic could change with the advent of more specific assay systems. For example, both asbestos fibres and diethylstilbestrol are carcinogens which do not cause mutations in bacteria. However, they have recently been shown to induce chromosomal changes such as non-disjunction in mammalian cells, probably as a consequence of binding to the spindle apparatus during mitosis (as reviewed by Barrett, 1986). The resulting gene imbalance could be an important step in the generation of aneuploidy which is frequently observed during tumour progression.

Multistage carcinogenesis

4.6 The concept of carcinogenesis as a multistep process was based on early studies in experimental animals by Berenblum (1941) and Mottram (1944). This work was reviewed by Foulds (1969) who postulated that tumours arise after a series of independent events. Epidemiological evidence in human populations (Peto, 1977), together with the results obtained using experimental systems, has confirmed and extended this concept. The process of tumorigenesis can be operationally divided into the stages of initiation, promotion and progression, a sequence most conveniently illustrated using the mouse skin model system in which the biological changes have been extensively investigated.

4.7 Initiation is thought to be a rare event, probably involving a somatic mutation which occurs in a small number of cells after a single application of a carcinogen to mouse skin at a low (sub-carcinogenic) dose. The development of a tumour takes place only after repeated treatment with another type of chemical which is not itself carcinogenic. Chemicals with this property are known as tumour promoters, the most potent being naturally occurring esters of the diterpene phorbol. They do not interact with DNA or cause mutations in mutagenesis assays, but have the capacity to promote the formation of benign tumours from initiated cells. Some of the benign tumours eventually progress to form carcinomas, a step which is not affected by continued phorbol ester treatment. Interestingly, chemical carcinogens can act at more than one stage of carcinogensis, since they have been shown to induce either initiation or progression (Hennings *et al.*, 1983). Other compounds which cause non-specific DNA damage by inducing active oxygen species can also act at either the promotion or progression stages. The development of maligant tumours is obviously extremely complex even in well-defined model systems in animals: many genes may be critical targets for chemical carcinogens and they can be mutated or activated at several different stages.

4.8 Direct evidence of initiation, promotion and progression in the natural history of human tumours is even more difficult to obtain. Progression, for example, is not a regular feature of most tumour types. It is perhaps best exemplified by the adenoma:carcinoma sequence in the common (non-familial) forms of colorectal cancer. A similar sequence, if it does occur, is less obvious in other high-incidence cancers arising in, for example, the breast or bronchus. Valuable information should however accrue from the use of modern techniques in molecular biology.

Critical DNA alterations in tumour cells

4.9 Until relatively recently one of the central problems in relating mutagenic effects to carcinogenesis was the lack of knowledge of the target genes. While a great deal of persuasive evidence was accumulated showing that carcinogenic chemicals can bind to DNA, either directly or after metabolic activation to 'ultimate carcinogens' (Conney, 1982), the nature of the critically altered genes remained unknown. Even more importantly, the question of whether the induced mutations were casual or secondary events in tumour development was not resolved.

4.10 One major advance in recent years has been the identification of a battery of normal cellular genes, now commonly referred to as 'proto-oncogenes', which were found to be altered in specific ways to form 'oncogenes' in tumour cells. Some of these oncogenes, of which at least 40 are now identified (Bishop, 1987), were first detected as integral components of transforming retroviruses isolated from rodent tumours. It transpired that these viruses had acquired their strongly transforming properties by picking up proto-oncogenes during passage through mammalian or avian cells. Sometimes these genes become altered, either in the transduction process or during the evolution of the virus, to forms which were strongly oncogenic. Molecular cloning has allowed the isolation of many of these genes from both normal and tumour cells, and approaches have been made to the central question of function.

4.11 The emerging consensus from these studies is that proto-oncogenes play essential roles in the control of normal cell growth and differentiation. Different classes of genes have been identified which code for growth factors or their cell surface receptors, or for proteins involved in the transduction of growth-inducing signals to the interior of the cell. Other proto-oncogene products located in the nucleus are probably involved in the initiation of gene transcription or replication. It is not difficult to envisage how disruption at any one of these links in the chain of events involved in controlling cell growth could lead to the aberrant behaviour of tumour cells.

Mechanisms of proto-oncogene activation

4.12 Of central importance in these developments was the discovery that DNA isolated from some human malignant tumours, but not that from normal cells, was capable of inducing the malignant phenotype when taken up by certain non-transformed mouse cell lines (Shih *et al,* 1981; Krontiris and Cooper, 1981). The molecular cloning techniques which have been developed within the last few years facilitated the rapid isolation and characterisation of that part of the DNA, the oncogene responsible for the transforming properties. The tumour gene (now know as c-*ras*) turned out to have only a single nucleotide substitution when compared with the same gene from normal cells (Tabin *et al.,* 1982; Santos *et al.*, 1982; Taparowsky *et al.*, 1982). This seminal observation has had widespread implications for studies on carcinogenesis and the possible role of mutagens in this process. Massive research effort has been invested in the detection of additional oncogenes, and in determining their mechanisms of activation in tumours.

4.13 Since similar DNA alterations have been noted in cells treated with mutagenic chemicals, it seems logical to expect that some proto-oncogenes may be the critical targets for activation by direct interaction with carcinogens. In some animal model systems, this expectation has already been fulfilled, since specific proto-oncogene mutations have been found in tumours induced by different classes of chemical carcinogens (reviewed by Balmain and Brown, 1988). The mutations were of the type predicted from the known metabolism and DNA-binding properties of the carcinogens used, suggesting that they occurred as a direct consequence of carcinogen treatmeant. Moreover, the causal nature of these mutations in tumour development is illustrated by the fact that introduction of the mutated oncogene, but not its normal counterpart, into suitable mammalian cells can reproduce the features of neoplastic transformation.

4.14 The mechanisms by which proto-oncogenes become activated to oncogenic forms in tumour cells include single point mutations, deletions, translocations of genetic material between chromosomes and gene amplification (Klein and Klein, 1985; Barbacid, 1987; Croce, 1987). In some cases, the main consequence of the genetic change is the overproduction of an otherwise normal gene product. This can occur by increasing the rate at which messenger RNA is produced from the gene (transcriptional activation) or at a later stage, by post-transcriptional stabilisation of the messenger RNA or the final protein product. Examples of both mechanisms have been seen in Burkitt's lymphoma cells and in mouse plasmacytomas induced by pristane treatment. In these tumours, the normal celluar *myc* gene, which is involved in the control of cell proliferation, is translocated to a different genetic locus (the immunoglobulin locus) which is highly transcriptionally active during B-cell development. The consequence can be an elevated level of the *myc* protein or the inability to switch off the gene at the appropriate time, resulting in uncontrolled cell proliferation.

4.15 A totally different mechanism of proto-oncogene activation is seen in human chronic myeloid leukemias which exhibit the 'Philadelphia chromosome'. In such cases, a piece of chromosome 9 carrying a proto-oncogene known as c-*abl* undergoes reciprocal translocation with a piece of chromosome 22. The c-*abl* gene becomes joined to a gene on chromosome 22 with the resultant production of a fusion protein encoded by both DNA sequences. The exact mechanism by which this qualitatively altered gene product exerts its transforming effects is not known.

Tumour suppressor genes

4.16 The existence of proto-oncogenes which can be activated by mutational mechanisms implies a positive role for mutagenic chemicals in tumour induction. There are however, other types of gene which contribute to neoplastic development only when deleted or inactivated. The best examples of this are seen in families which have a genetic predisposition to particular types of cancer. Retinoblastomas and osteosarcomas develop in children who inherit a defective copy of chromosome 13 from one parent. The defect has been shown to involve a deletion or mutation in a specific gene which has recently been identified (Friend *et al.*, 1986). Tumours arise when the normal copy of the same gene on the other chromosome is lost or mutated in early childhood. This demonstrates that the gene, when present in a functional state, has suppressive effects on the development of tumours. A similar situation can arise in children with the Tay Sacs familial variant of Wilms tumour. Genes on chromosome 11 are frequently lost in tumours from such patients (Koufos *et al.*, 1985), and it is possible, although not yet proven, that predisposition is determined by the inheritance of a defective allele at the same locus.

4.17 These special examples could be indicative of a more general role for tumour suppressor genes in non-hereditary forms of cancer. Loss of genetic heterozygosity at a number of chromosomal loci has been seen in a variety of human tumours. Re-introduction of a normal copy of chromosome 11 into tumour cells by microcell fusion can lead to suppression of the tumorigenic phenotype (Saxon *et al.*, 1986). There is evidence for the existence of other tumour suppressor genes in different human chromosomes, suggesting their involvement in a wide variety of tumour types (Stanbridge, 1985). Since, in principle, it is much easier to inactivate the function of a suppressor by mutation or deletion than to activate a proto-oncogene, the former may constitute important targets for carcinogenic chemicals at some stage of tumorigenesis.

Implications of mutagenicity testing for regulation of carcinogens

4.18 A wide spectrum of mutational changes has been implicated in the induction of neoplasia. It is prudent to assume that any chemical capable of causing mutations *in vivo* in mammals is a potential carcinogen. It should be noted that the expression of carcinogenic potential can vary markedly between species, and mutagenicity data cannot predict which species, if any, will be susceptible for tumour induction. Nor can the results of mutagenicity tests give quantitative information on the carcinogenic potency of a chemical or the type of tissue in which tumours will arise. A further limitation is that not all potential carcinogens will be detected by mutagenicity assays. A negative result will not constitute proof that a compound is non-carcinogenic.

4.19 Our increasing knowledge of the molecular mechanisms of cancer induction may eventually lead to the development of a battery of tests which will provide a more realistic basis for prediction of carcinogenic potential in man. Such tests may be based on the capacity of particular chemicals to induce gene amplification, gene conversion or non-disjunction in mammalian cells or to cause specific activating mutations in defined proto-oncogenes. There is also a requirement for new means of detecting non-genotoxic carcinogens or tumour promoters, for which there are no adequate short term tests available at present. Although the development of such tests is at a very preliminary stage, it is important for the prediction of carcinogenicity to employ genetic end-points relevant to different stages of carcinogenesis, rather than concentrating exclusively on systems which are heavily weighted towards the detection of initiators or point mutagens.

Future prospects

4.20 The impact of molecular biology and the discovery of proto-oncogenes has led to a major advance in the understanding of carcinogenesis at the molecular level. However, the complexity of the relationships between different oncogenes is only beginning to be understood. Cell growth is controlled by a complex network of pathways involving different gene families, each member of which is potentially capable of activation by chemical carcinogens. Chemically induced tumours or transformed cell lines have been found which show specific genetic changes involving growth factor receptors (*neu*), signal transducing systems (*ras*) or nuclear DNA binding proteins (*myc*). Some alterations involve mutations in the structural gene, while others involve regulatory elements. It is to be expected that further work will reveal examples of activating mutations or rearrangements in many other types of proto-oncogene. The information obtained will have relevance to studies on mutagenesis, in particular in the identification of specific adducts or mutations which contribute to the transformed phenotype, to the control of cell growth and differentiation and finally to the elucidation of the role of environmental carcinogens in the development of human cancers.

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5. Genetic and partly genetic diseases of man: types, frequencies and mutation rates

Introduction

5.1 Before any untoward effects on human populations of increases in mutation rates, which could be caused by environmental mutagens, can be considered, it is necessary to have sound knowledge of the existing backgrounds of mutations detrimental to man. The object of this chapter is to review what is known of this background mutational load. Changes in genetic material, ie mutations, contribute significantly to human congenital abnormality and to disease manifest in early life, or that which makes its first appearance during early adulthood or later. For reasons that will be apparent, the total contribution which genetic change, however it may originate and be maintained in the population, makes to human disease and disability is not precisely known, and in any case is variable in different populations but is estimated to be of the order of 1.6% (UNSCEAR, 1986). However, this is a conservative estimate for the burden imposed by chromosome disorders (eg Down syndrome) and by diseases caused by single genes of large, and usually obvious, effect (eg cystic fibrosis). If other diseases with a genetic component (eg diabetes mellitus, mental illness and congential abnormalities) are included, the genetic contribution to frank disease can be estimated to be greater than 5.3% (Baird *et al.*, 1988: see Table 5.10). In addition, there are many more subtle genetic influences, often immunological or biochemical, that affect human characteristics which predispose those who carry such traits to a variety of untoward pathological responses to environmental influences and thus to disease (eg various allergies). At present, it is not possible to quantify this part of the potential genetic contribution to ill-health, but evidence suggests that it is substantial. However, there are some categories of genetic change that are more exactly documented than others and thus their contribution to human abnormality can be more accurately assessed. For this reason, as well as for reasons connected with the origins and maintenance of mutational changes, it is useful to consider these changes under four main categories:

(a) Chromosome abnormalities.

(b) Defects and diseases due to single genes of large effect.

(c) Congenital abnormalities with evidence of a sizeable genetic contribution to their origin.

(d) Complex disorders in which genetic influences play an important role and various other diseases in which there is a variable genetic influence.

5.2 In general, the influence of any increase in mutation rate will be uneven in these different categories. In (a), one is dealing with numerical and with structural chromosome alterations and most are new mutations. The mechanism resulting in an alteration in chromosome number is generally non-disjunction and its ultimate causation in man is unclear. It is often dependent on maternal age (suggesting non-disjunction in oogenesis) but meiotic non-disjunction can also occur in the male germ lines, and may even involve both meiotic divisions sequentially. The mechanisms of structural chromosome changes (rearrangements) are often, if not always, chromosome-breaking events followed by misplaced reunion.

5.3 It has been estimated that of human pregnancies recognised by a missed menstrual period, about 15% end in miscarriage. With the use of hormone assays, permitting earlier detection of pregnancies that would have otherwise gone unrecognised, an even higher fraction of those pregnancies was found to terminate without issue, yielding a combined miscarriage rate of 50% (Sperling, 1984). Of all human conceptions, at least 5–10% are chromosomally abnormal and a high proportion of these will result in miscarriage due to lethal genetic imbalance. The exact frequency of this is not easily ascertained, but it probably lies between 15% and 20% of recognisable pregnancies. Thus, a large proportion of these mutations remain hidden, making it difficult to detect moderate but still significant increments of these mutations. Among survivors at birth, early mortality is high in some cases.

5.4 In (b), effects involving single genes may be a consequence of point mutations involving single DNA bases, or large changes, including deletions, within the gene; these changes are not normally detectable with conventional microscopy. In this group, immediate effects of mutagens would be evident as increased frequencies of those detrimental dominant diseases and those conditions that are more obvious near birth. Naturally, when considering germinal mutations, account would have to be taken of the long generation time of man, of the difficulties of monitoring human populations, and of knowing accurately the baseline of existing detrimental mutations.

5.5 In (c) and (d), the effects are often determined by the action of many genes and depend on strong interactions with the environment, that is the effects are multifactorial. It is difficult to predict what the quantitive influence of an increase of mutations might be on the incidence and prevalence of the various traits and disorders mentioned in both these categories. *Prima facie*, the frequency of these conditions would appear to be relatively insensitive to mutational changes, unless they are of a very high order, or gradually accumulating over the generations. However, it is possible that the impact resulting from mutational changes of genes which generally act in a multifactorial context may be far from negligible (Vogel, 1979)

a. Chromosome abnormalities

5.6 The normal chromosome complement in the somatic cells of man consists of 46 chromosomes (ie 23 pairs), one in each pair of maternal and paternal origin. The normal chromosome complement in human germ cells (ova and spermatozoa) is 23, ie one-half of the somatic complement. Each chromosome can be identified under the light microscope with precision using special staining methods which result in specific 'banding' (see Figures 5.1, 5.2). The number and different sizes of alternating dark and light bands which can be revealed give each chromosome a characteristic and individual appearance and define a number of segments within each chromosome. The chromosome complement, whose orderly arrangement is called a 'karyotype' consists of 22 pairs of non-sex chromosomes (autosomes) and a pair of sex chromosomes. Each chromosome has a constricted portion, the centromere, which divides the chromosome into two arms—a short arm and a long arm, designated p and q respectively. The autosomes are numbered sequentially from 1 to 22, from largest to smallest. Normal females possess a medium-sized pair of sex chromosomes called X chromosomes; normal males possess one X chromosome and a much smaller sex chromosome, called the Y chromosome. In normal circumstances, sex development in the embryo depends on the presence (maleness) or absence (femaleness) of this Y chromosome, whose main function appears to be that of inducing testis development. By contrast, the X chromosome carries a large number of genes which act on many aspects of somatic development and function ranging from, for example, blood clotting to intellectual function. Genes on the X chromosome are said to be X-linked; characteristics which they determine are called sex- (or X-) linked and are transmitted in a pattern which differs from that of autosomal genes. A further important characteristic of X chromosomes is that only one X chromosome functions when more than one, eg two in normal females, is present in the somatic cells. It follows that only one X chromosome reveals it genes at a cellular level. The process of inactivation of one of the X chromosomes in each somatic cell occurs early in development, probably not later than the sixteenth day of development when the embryo consists of less than 5000 cells, and is random, affecting either of the two Xs in different cells of the same individual (known as 'Lyonisation' after Mary Lyon, the discoverer of this phenomenon). Thus, in a normal female, some somatic cells show the activity of X-linked genes inherited from her mother, while others display those from her father. This is known as mosaicism.

5.7 By international convention, the chromosome complement of an individual is described by the total number of chromosomes, followed by the description of the sex elements. Thus a normal female is described by 46,XX and a normal male as 46,XY. The chromosome constitution in a normal human ovum is 23,X whereas normal males produce equal numbers of spermatozoa with 23,X or 23,Y chromosome constitutions.

5.8 Chromosomal abnormalities, probably the most clearly defined of all human mutations, account for at least 60 different recognisable syndromes in survivors at birth (Schinzel, 1983). Although considered mutations in a general sense, they are more specifically genome and/or chromosome mutations; ie numerical and/or structural chromosome abnormalities. The best known numerical abnormalities are the autosomal and sex chromosomal trisomies

Figure 5.1: *Human karyotype (schematic)*

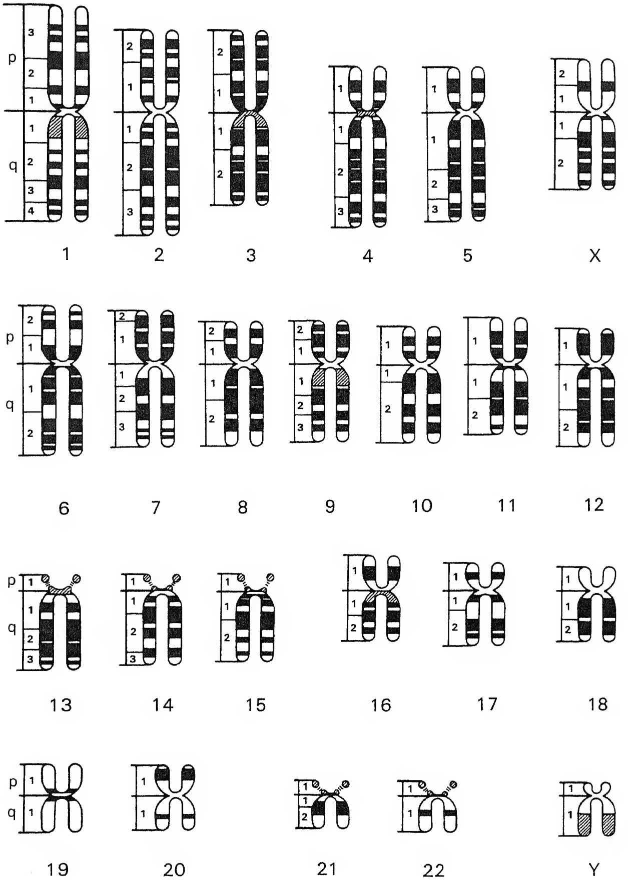
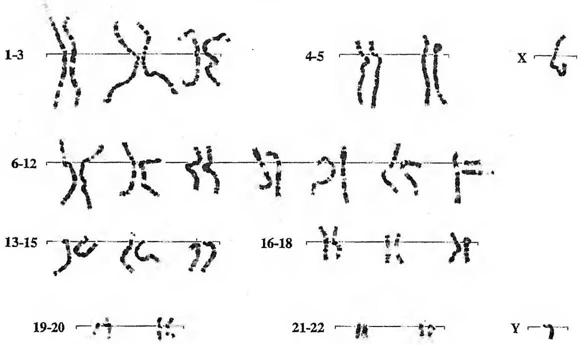


Figure 5.2: *Human karyotype (photomicrograph)*



which cause the clinical syndromes of Down (trisomy 21), Edward (trisomy 18), Patau (trisomy 13), Klinefelter (sex chromosomes XXY), the XYY and XXX sex chromosome abnormalities and the XO sex monosomic condition (Turner syndrome). In the XO (45,X) condition, there is only one sex chromosome, as a result of loss of either an X chromosome (maternal or paternal) or the paternal Y. Other numerical chromosome abnormalities are known in survivors, some with more than one extra sex chromosome, for example, 48,XXXX or 49,XXXXX, or an extra autosome plus extra sex chromosome, for example, trisomy 18 and XXY or XXX (karyotype 48,XXY or XXX+18). Most other imbalanced chromosome abnormalities are a consequence of structural chromosome changes which result in duplication of parts of chromosomes (partial trisomies) or deficiencies (partial monosomies), or both together (duplication-deficiency). They are the derivatives of translocations of segments between chromosomes or rearrangements, including inversions within chromosomes, simple deletions, or ring or isochromosome formation. An isochromosome results for mis-division, transverse instead of longitudinal, at the centromere; it consists of a duplicated short (p) or long arm (q) of a chromosome with the other arm missing.

5.9 In addition, there are chromosome abnormalities of structure in which no apparent imbalance results, such as balanced translocations in which there is an exchange of segments between non-homologous chromosomes. When the translocation involves the acrocentric chromosomes, namely numbers 13, 14, 15, 21 and 22, the process is sometimes referred to as 'centric fusion' and this type of translocation as a 'Robertsonian translocation'. The fragment formed by the short arms of the two chromosomes is lost. A receiprocal translocation involves the exchange of chromosome material distal to breaks in one of the arms of two non-homologous chromosomes. A person carrying such a balanced chromosomal rearrangement will usually be normal since no obvious chromosomal material has been lost. However, such individuals have an increased risk of producing gametes with an unbalanced chromosome complement which can result in spontaneous miscarriage or in a child with a congenital abnormality. The precise detection and classification of structural chromosome mutations, such as translocations, inversions, deletions and duplications has been made easier and more precise by chromosome banding techniques.

5.10 Chromosome studies of some 56,000 liveborn infants in many countries (Hook and Hamerton, 1977) detected a frequency of abnormalities of about one in 150 (Table 5.1 based on about 43,000 births). Most chromosomal abnormalities are numerical, of which the most common is trisomy 21 (Down syndrome), but sex chromosome trisomy (XXX, XXY or XYY), as a group, is somewhat more frequent than autosomal trisomy. Structural chromosome abnormalities constitute about one third of the total (affecting about one in 500 newborn infants) and of these, about 13% are unbalanced, though a somewhat higher figure is sometimes quoted. Of the structural abnormalities, between 15% and 22% are new mutations, while the remainder are inherited, somewhat more often from the mother.

5.11 Most autosomal abnormalities are lethal prenatally or perinatally, as spontaneous abortions or as later fetal (stillbirths) and early neonatal deaths (grouped together as perinatal deaths). Some autosomal abnormalities with imbalance are compatible with survival to birth but are generally lethal in early postnatal life. Trisomy 21 is an exception. Chromosome abnormalities are found in more than 5% of neonatal deaths, in 4.5% of fresh stillbirths and in over 10% of macerated stillbirths. However, chromosome abnormalities are particularly frequent in spontaneous abortions, 30% to 50% or more of which are chromosomally abnormal (see Table 5.2; Creasy, 1977; Boué and Boué, 1978). The variation reported in different studies depends largely on the gestation age of the abortuses. Pooled data from a number of major chromosome studies of spontaneous abortion on almost 2000 chromosomally abnormal abortuses are shown in Table 5.3. A small proportion of these were investigated by banding methods and allow some general conclusions to be reached about the distribution of the specific autosome trisomies.

5.12 From these data, it is possible to arrive at approximate prevalance figures for various chromosome abnormalities in early conceptions based on, the frequency of spontaneous abortion, the proportion of chromosomally abnormal miscarried fetuses and, the proportion of the different chromosome abnormalities found in them. The frequency of spontaneous abortion is estimated to lie at between 15% and 20% of all recognisable pregnancies and that of chromosomal abnormality among abortions is probably about 40%, though this figure is influenced by the gestation age of the miscarriages studied.

5.13 On this basis, it seems probable that 6% or more of all early conceptions which lead to a recognisable pregnancy are chromosomally abnormal. If very early conceptions, detectable only by raised hormone levels, are included, this figure could be 8.5% or higher. The appropriateness of this estimate seems to receive confirmation from the chromosome findings in abortions induced for socio-economic reasons, from studies on chromosome abnormalities detected in human spermatozoa (Martin *et al.*, 1983; Brandriff *et al.*, 1984; Kamiguchi and Mikamo 1986) and in human pre-embryos produced by in vitro fertilisation (Angell *et al.*, 1986; Rudak *et al.*, 1984). It can be further estimated that about 1.2% of all recognisable pregnancies could be 45,X, about 1% triploidy and 3% autosomal trisomies (almost 1% trisomic for chromosome 16 and about 0.45% trisomic for chromosome 21—Down sydrome). Some 90% of chromosomally abnormal fetuses are rejected as spontaneous abortions, some (triploidy and 45,X) more frequently than others (for trisomy 21, of which about two thirds abort and some are eliminated as stillbirths), and some hardly at all (sex chromosome trisomies, XXX, XXY, XYY; some of these are lost as perinatal deaths). The chromosomal mutations, particularly the numerical ones, seem suited to monitoring at birth. Obviously, there are problems with most trisomies because, the frequency of these is related to the age-structure of the population of reproductive women and, because of the high proportion to be found in spontaneous abortions and prenatal deaths. Thus it follows that, chromosome abnormalities at birth represent only the tip of an iceberg and, differences in birth incidence cannot be taken automatically to reflect differences in the frequency of non-disjunctional events occuring prior to conception; they could well be due to non-genetic factors affecting prenatal mortality. For this reason alone, attempts to monitor this type of mutation would be beset with difficulties.

b. Defects due to single genes of large effect

5.14 Three main patterns of monogenic Mendelian modes of inheritance are recognised; autosomal recessive, autosomal dominant and sex-linked (or X-linked), which may also be recessive and dominant. By 1988, 2208 'proved' mutant phenotypes had been catalogued with an additional 2136 listed as 'probables'. Of the former, 1443 were classified as autosomal dominant, 626 as autosomal recessive and, 139 as X-linked, with a similar proportional distribution amongst the probables (McKusick, 1988). This total number of diseases and conditions is considered to represent only a small proportion of the many gene loci in man capable of causing effects detrimental to health.

5.15 The mutations within, or, affecting the expression of, single genes responsible for inherited defects in man encompass a range of different alterations at the DNA level. At the extreme, a mutant phenotype may be a consequence of the complete loss of a gene (eg in some inherited childhood cancers such as Wilm tumour or retinoblastoma), or partial loss rendering the gene, or its altered product, non-functional (eg in some cases of haemophilia or muscular dystrophy), or of smaller changes within the gene which result in an abnormal protein product with reduced or abnormal function (eg sickle cell anaemia). Within any group of diseases, (eg the haemoglobinopathies), a spectrum of different mutational changes can be indentified. In this latter example, different mutant alleles for the genes coding for the various globins have been shown to have arisen from single DNA base alterations (transitions or transversions), or frameshift changes, consequent to deletion or insertion of one or more base or resulting from unequal crossing-over. In addition, mutants due to initiation or termination codon mutations, 'regulation-box' mutation, poly A addition site mutation and, fusion genes have been described. An analysis of the molecular basis for the haemoglobinopathies has shown that human genetic disease is exceedingly heterogeneous at the molecular level.

5.16 The recent application of molecular biology to study inherited defects in man has led to numerous advances in our understanding of the nature of the mutations and in many instances, in the complete isolation and characterisation of both normal and mutant genes. In addition, DNA markers, linked to genes of interest, have been identified and are now used in diagnosing the presence or absence of mutant genes know to be responsible for human disease. In many cases, the structure and/or the function of the mutant gene causing the disease is unknown (eg Huntington's disease), but the availability of tightly linked indentifiable and detectable DNA sequences (probes) closely adjacent to the abnormal gene, in a family in which the abnormal gene is known to be segregating, enables the presence or absence of the abnormal gene to be established with varying degrees of probability. Such markers are now in routine use for the antenatal diagnosis of a variety of inherited diseases (eg cystic fibrosis, sickle-cell anaemia and Duchenne muscular dystrophy) and rely on an analysis of DNA from small samples of chorionic villi or exfoliated fetal cells (amniocytes).

5.17 The prevalence in the population of different countries of diseases caused by single-gene mutation is imprecisely known, probably due to different methods of ascertainment. However, variation in prevalence is sometimes due to an actual variable prevalence of some genetic diseases in different populations (see Table 5.6; paragraph 5.34). The main reasons for the variability of the estimates of the overall load of genetic disease are the inclusion or exclusion of disorders which cause relatively little inconvenience in early adult life, as with some autosomal dominant diseases, eg polydactyly, or of conditions which do not represent disease entities *per se* but nevertheless play a part in the origin of diseases that manifest mostly only in adult life, eg familial hypercholesterolaemia and α1-antitrypsin deficiency. These conditions have not been tabulated and are often considered as 'traits', albeit potentially detrimental, rather than disease entities.

5.18 It must be stressed that most human genetic diseases are genetically heterogeneous (Harris, 1974). This may mean that, in different families, the same disease is caused by different alleles at one locus, or the heterogeneity can result from mutations at different gene loci which are normally responsible for the production of the same, or different, enzyme-proteins. These enzyme-proteins subserve similar functions and, their alteration causes disorders which are not yet clinically distinguishable from each other. Thus, 'mimic' genes seem to be a feature of at least some human genetic diseases. Additionally, heterogeneity of genetic disease results from the activity of the whole genetic background on the loci under scrutiny and from the activities of environmental factors.

5.19 Table 5.4 is a conservative list of the incidence in populations, related to 1000 livebirths, of the major autosomal dominant diseases. The miscellaneous group, 'other', is likely to be an underestimate. It is worth noting that of these conditions, neurofibromatosis, the gene for which has recently been shown to be located on the long arm of chromosome 17 (Barker *et al.*, 1987; Seizinger *et al.*, 1987) and which appears to be relatively common, shows little evidence of genetic heterogeneity. Somatic mutation may be responsible for a proportion of cases which, incidentally, has nearly the highest estimate of germinal mutation rate of any of the known dominants (see Table 5.12). The overall incidence of autosomal dominants is thus between 2.08 and 2.79 per 1000 births if one includes the relatively common hereditary spherocytosis and adult polycystic disease of the kidneys. Several autosomal dominant disorders have not been included in Table 5.4 because of under ascertainment in many studies. If otosclerosis, the adult onset form (Morrison, 1967), which in about one-third of cases appears to be inherited in an autosomal dominant manner, was included, the incidence of autosomal dominants would rise a further 1.0–1.5 per thousand.

5.20 It must be emphasised that some of the figures are underestimates due to the difficulties of ascertainment, particularly of those dominants with delayed manifestation in late adult life. For example, with Huntington's disease, Baird *et al.*, (1988) found an incidence rate of 0.016 per 1000 livebirths. The average age of onset of Huntington's disease is 41 to 45 years and the actual incidence is closer to 0.4 per thousand (Vogel and Motulsky, 1986).

5.21 It is important to note that some of these relatively common dominant diseases of delayed onset in late adult life are unlikely to be maintained at their high level of prevalence by a high mutation rate, but have been accumulated in the human population throughout evolutionary time. Technical reasons make estimation of the actual mutation rates (see Table 5.12) difficult and imprecise.

5.22 Deafness and blindness are comprehensive categories which, like severe mental retardation (see Tables 5.5, 5.6 and 5.8), include conditions of varying genetic origin as well as some of environmental source. In Tables 5.5 and 5.6, attempts have been made to include only the genetic components. It has been estimated that there are at least 34 'mimic' genes for deafness (but there might be 100), and probably more for blindness. For deafness, it is assumed that there are five autosomal dominant genes, 15 to 25 autosomal recessive genes and four X-linked genes. The corresponding figures for blindness gene loci are 15, 23 to 35 and, nine (Cavalli-Sforza and Bodmer, 1971).

5.23 A common condition not listed among the autosomal dominant disorders is mongenic familial hypercholesterolaemia (Fredrickson type II), which is believed to have an incidence of two per thousand in the population (Vogel and Motulsky, 1986). It is related to ischaemic heart disease, which usually occurs in the second decade of life in the homozygous mutants; in half the heterozygotes, some manifestation of coronary artery disease is present by 50 or 60 years-of-age. The other autosomal dominant disorders of lipid metabolism appear to be even more common (familial hypertriglyceridaemia and mixed hyperlipidaemia). Another condition which is relatively common and clearly heterogeneous is gout (Kelly and Wyngaarden, 1983) with a population incidence of about three per thousand. Another relatively common trait is $\alpha 1$-antitrypsin deficiency, which is related to specific alleles present at the protease inhibitor (Pi) locus. In European populations, it is estimated that one in 1500 to one in 3000 livebirths can be affected with antitrypsin deficiency (Harris, 1975; Fagherhol, 1976). As adults, some 15% to 30% of antitrypsin deficiency individuals develop serious pulmonary emphysema in response to environmental urban pollutants and smoking. The addition of these and similar conditons and traits could increase considerably the estimate of total incidence of autosomal dominant disorders.

5.24 Table 5.5 lists some of the more common autosomal recessive diseases in Caucasian populations and again, as with autosomal dominant diseases, the group 'others' is certainly an underestimate. Again, the variation in the estimated frequencies of autosomal recessive diseases is due to the sampling of different populations with different genetic backgrounds, the variation due to sample size, and also the different methods used to estimate the frequency. As an example, gene frequencies, and thus disease frequencies for rare recessive conditions, can be calculated by using an estimate based on the frequency of first-cousin marriages. This method provides only a crude approximation, as no account is taken of contribution of more than one locus, of more than one allele (in the case of abnormal compound heterozygotes), or of less strict degrees of consanguinity. The application of biochemical techniques which has made the recognition of autosomal recessive disorders easier has allowed the recognition of many examples of enzyme-protein variation, some very rare and others reaching polymorphic frequencies. Thus, suxamenthonium sensitivity, for example, which is found in about 0.5 per 1000 Europeans, is due to the presence of mutant alleles which result in the production of a serum cholinesterase with impaired activity (Harris, 1975).

5.25 Studies at the molecular level have established the chromosomal location of some important recessive mutant genes and have provided useful DNA probes for carrier detection, antenatal diagnosis and diagnosis of homozygous affected states (eg cystic fibrosis; Knowlton *et al.*, 1985; Wainwright *et al.*, 1985; White *et al.*, 1985). Moreover, in some instances (eg phenylketonuria; DiLella *et al.*, 1986), the gene has been isolated and the molecular defects in mutants characterised. It should be emphasised, however, that many autosomal recessive conditons are heterogenous and that their prevalence may differ markedly between populations of different ethnic origins, eg cystic fibrosis is relatively common in most Caucasian populations but 30 to 40 times rarer among Africans and Orientals (see Table 5.6).

5.26 Similar ethnic variability is noted in Tay-Sachs disease, which has a frequency of about one in 3000 in Ashkenazi Jews and about a thousand times less in other Jews and in Gentiles. The metabolic defect involves a lysosomal enzyme defect (hexosaminadase A), which causes a lipid-storage disease fatal to homozygotes in early childhood. A reduced enzyme level is readily detectable in heterozygous carriers and successful carrier screening programmes have been set up in many countries (Kaback, 1977). Tay-Sachs disease, as well as some others showing these large population frequency differences, must be maintained in the relevant populations at their high frequency levels largely by means other than recurrent mutation or founder effect. It has been postulated that geographically widespread deleterious genes present in high frequencies have a compensating advantage. In the case of Tay-Sachs disease, evidence suggests this advantage is resistance to tuberculosis.

5.27 Table 5.7 lists the approximate incidence at birth of the commoner sex-linked recessive diseases. Many others are very rare; for example, Lesch-Nyhan disease, due to a defect of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT), is between one and two orders of magnitude, rarer than some of those quoted in Table 5.7 (Seegmiller, 1976), yet this disorder is heterogeneous. The gene coding for the enzyme has been shown to have several different mutations within the gene (Caskey, 1988). Heterogeneity is also evident for the X-linked gene coding for the enzyme glucose-6-phosphate dehydrogenase (G-6-PD), a defect responsible for an environmentally triggered haemolytic anameia. More than 20 different forms (alleles) of G-6-PD gene are known to exist, many of which reduce G-6-PD activity (Harris, 1975). Two forms, one in African and the other in Mediterranean populations, reach polymorphic frequencies, probably because the deficiency of the enzymes in the red cells acts as a protective mechanism for carriers of the gene in malarial zones. Among the more common of the sex-linked disorders, mention should be made of Duchenne muscular dystrophy (DMD), which at a molecular level turns out to be a very large gene and one in which much progress has delineated the range of different mutations within the gene responsible for this serious disease (Koening *et al.*, 1987). A protein (dystrophin) encoded by the DMD gene has been identified, attached to the surface membrane in normal muscle; it is undectable in muscle from patients with DMD. Another X-linked disorder, the fragile X syndrome (Martin Bell syndrome), which appears to be one of the more common causes of mental retardation in males (with a prevalence approximately to that of Down syndrome) is the subject of active research at the molecular level.

5.28 The question of dominance or recessivity of X-linked conditions is complex due to the random inactivation in female cells of one X chromosome, with the consequent activity of its homologue only. Because of this, intermediate female manifestations on many X-linked 'recessive' disorders are not unusual. In the few X-linked abnormalities classified as dominant (incontinentia pigmenti, orofacio-digital syndrome), it seems that affected males die soon after conception with consequent deficiency of males in the families. Only genetically and phenotypically normal males survive and diseases are transmitted from mother to daughter. Non-fragile X-linked mental retardation, like blindness or deafness, is a heterogeneous category and the frequency of the occurence of cases in this group is only an approximate estimate.

5.29 In summary, individual human monogenic diseases have birth frequencies ranging from about one in a hundred thousand (10^{-5}) to about one in a million (10^{-6}). Diseases which are due to mutant genes with a specific single base substitution can have frequencies of the order of one in a billion (10^{-9}). Conversely, there are genetic conditions with birth frequencies around one in 1000 or even higher. Taking an average fequency of single gene disease of one in 10^{-5} to one in 10^{-6} births and relating this to more than 1000 genetic disorders so far catalogued, the overall incidence at birth of monogenic diseases could lie between 0.1% and 1.0%. The totals cited in Tables 5.4, 5.5 and 5.6 are within these limits. In the United Nations Scientific Committee's latest assessment of genetic risks from radiation (UNSCEAR, 1986), an estimate of 1.25% has been used as the combined incidence of serious dominant and X-linked conditons. As already stressed, these figures vary when different populations are considered, and some examples of the variable frequencies of genetic diseases are given in Table 5.6, while the ethnic variability of some malformations is listed below. There are several possible reasons for the variation in Table 5.6, but one of them, when dealing with recessive conditions, may reside in a selective advantage possessed by heterozygous carriers, to which we have already referred when discussing X-linked G-6-PD and Tay-Sachs disease (see paragraph 5.27).

5.30 In this situation, the selective advantage enjoyed by the carriers in a particular environment leads to a persistence in the population of the recessive gene, which is detrimental in the homozygous state and indeed may lead initially to selective increment in gene frequency over a period of time. Such a selective advantage can operate only as long as the particular environmental conditions pertain. The classic, and still only well understood, example of selective advantage is provided by the gene encoding sickle-cell anaemia. The homozygous carriers of this gene die or fail to reproduce because of sickle-cell anaemia. However, the much more numerous heterozygous carriers (those with sickle-cell trait) resist malaria better than normal individuals. For this reason, the sickle haemoglobin gene frequency has become as high as 10% in certain populations where malaria exercises a selective pressure.

5.31 A similar situation exists for thalassaemia in respect of its widespread polymorphism and relationship to a malarial environment. Again, thalassaemias are heterogeneous disorders of synthesis of haemoglobin which result from the reduced rate of production of one or more of the globin chain of haemoglobin with consequent imbalance of the types of haemoglobin synthesised. There are two main classes of thalassaemias, the α and the β, each with several distinct genetic types. The diseases which they produce can be relatively common in Mediterranean peoples (Italians, Greeks, Turks and Iranians), among whom the heterozygous carrier state (thalassaemia minor, thalassaemia trait) can reach frequencies of 10%–20% and the number of those affected by the severe disease produced by the homozygous state of the abnormal gene may amount up to 1% of all births. In Great Britain, homozygotes for thalassaemia are found among persons and their decendants whose country of origin, in more than half the detected cases, was Cyprus (Modell *et al.*, 1980).

c. Congenital abnormalities

5.32 The incidence of the more common congential abnormalities is listed in Table 5.8. Minor and relatively unimportant conditions like accessory ear tags, 'postural' club-foot, or birth marks are excluded, but glandular hypospadias is included.

5.33 Several congenital abnormalities appear to be multifactorially determined, often with an important environmental component. Evidence for this view is probably best for pyloric stenosis and congenital dislocation of the hip, and good for the major neural tube defects. The evidence is less, for example, for certain types of cardiovascular abnormality.

5.34 For some of the congenital abnormalities listed in Table 5.8, there is considerable regional or racial variation. For example, in the British Isles, neural tube defects (anencephaly and spina bifida) have a higher incidence in the north and west than in the south and east. During the mid 1960s, figures above four per 1000 births for anencephaly and a similar figure for spina bifida have been observed in Northern Ireland (Elwood and Nevin, 1973) and in South Wales (Richards *et al.*, 1972), whilst, at this time, the figure for each defect was only about 1.5 per 1000 births in London (Carter and Evans, 1973). In the British Isles, over the past decade, there has been a substantial decline in the incidence rate of neural tube defects. For example, in Northern Ireland, the incidence rate has fallen from 7.6 per 1000 births in 1964 to 3.6 per 1000 births in 1980 (Nevin, 1983). The causes of the decline are unknown. Antenatal diagnosis and selective abortion of affected pregnancies and prevention of recurrence of neural tube defects by periconceptional vitamin supplementation accounts for a small proportion of the decline in incidence rates (Nevin, 1983). The incidence of club-foot in Orientals is about half that among Caucasians, whilst in Hawaiians and the other Polynesians, it appears to be six to seven times more frequent. The prevalence of serious mental handicap, two per thousand (Penrose, 1963; Polani, 1973) is based on estimates in school children, and excludes Down syndrome and the groups in Tables 5.5 and 5.6. When mental handicap resulting from severe infection and trauma is excluded, at least 12% of cases of severe mental handicap could be attributed to the action of recessive genes (see Tables 5.5 and 5.6): in addition, some genetically determined cases will be due to dominant genes and some of polygenic origin. Altogether, it is considered that at least 350 autosomal loci are responsible for mental handicap and that approximately one-third of normal persons are heterozygous for gene(s) for severe mental handicap (Motulsky, 1976). Congenital instability of the hip is five to 20 times more common in the newborn than the estimate given in Table 5.8.

d. Other diseases in which there are genetic influences

5.35 Mental illness has a population prevalence of about 1.5% and with epilepsy represents a considerable burden of ill-health (Baird *et al.*, 1988). Another relatively common condition is diabetes mellitus. Table 5.9 lists some conditions in which genetic factors seem to be important but play a variable role. To these could be added the apparent contribution of genetic influences to ischaemic heart disease (paragraph 5.22), to a range of diseases associated with inherited HLA haplotypes (eg ankylosing spondylitis, rheumatoid arthritis, psoriasis, multiple sclerosis, coeliac disease—Crumpton, 1987) and to cancer.

5.36 At the cellular level, human cancer is a genetic disease, although the genetic alterations associated with it usually arise in somatic cells later in life and are not inherited (a number of alterations involving human oncogenes have been characterised—Bishop, 1987). There are, however, many notable exceptions in which a clear predispositon to cancer is an inherited trait. Examples include the childhood cancers, retinoblatoma and Wilm tumour, which involve mutations of specific genes on chromosomes 13 and 11, respectively (Fung *et al.*, 1987; van Heyningen *et al.*, 1985). Genes have also been located on chromosomes 3, 5, 10, 11, 17 and 22 for carcinoma of lung, familial adenomatous polyposis, thyroid cancer (MEN II), multiple endocrine neoplasias (MEN I), neurofibromatosis and bilateral acoustic neuroma, respectively (Brauch *et al.*, 1987; Bodmer *et al.*, 1987; Mathew *et al.*, 1987; Simpson *et al.*, 1987; Barker *et al.*, 1987; Seizinger *et al.*, 1987). In addition to these genes that directly predispose to specific cancers, there are many which are responsible for inherited conditons associated with an increased cancer risk (eg xeroderma pigmentosum, Bloom Disease, Fanconi anaemia, ataxiatelangiectasia—Cleaver, 1984).

5.37 As for the manner in which genes contribute to the origin of schizophrenia, various models have been proposed, from the single monogenic to the multigenic. The monogenic model has two alternatives—autosomal dominant or recessive. A threshold for the origin of the disease in those carrying the relevant gene(s) is incorporated in some of the models. In all models, a sizeable environmental component is accepted as a triggering factor of the illness (Slater and Cowie, 1971). The manner in which genes contribute to the origin of the affective psychoses is equally uncertain, but again the existence of a genetic component is not in doubt. For the bipolar affective disorders (manic depressive illness), major autosomal monogenic, as well as dominant X-linked inheritance (Mendlewicz and Rainer, 1977) has been proposed. The association with an X-linked locus has been confirmed (Baron *et al.*, 1987) and other reports have demonstrated linkage of a dominant gene to DNA markers on chromosome 11 (Egeland *et al.*, 1987) and in other families, dominant gene(s) on other autosomal chromosomes (Hodgkinson *et al.*, 1987; Detera-Wadleigh *et al.*, 1987). Studies on Alzheimer disease, a common dementia affecting from 1% of persons aged 65, to 15% of those aged over 85, suggests that a rare familial form may be coded for by a single dominant gene on chromosome 21 (St George-Hyslop *et al.*, 1987; Kang *et al.*, 1987). However, in a larger survey of Alzheimer disease in 15 families, no evidence for linkage was found between familial Alzheimer disease and chromosome 21q21 markers (Schellenberg *et al.*, 1988). As already stated, it is impossible to predict the effect on the incidence which an increment of the mutation rate of the genes responsible for the genetic component of the psychoses and the other conditons in this heterogeneous group of conditions would have. Nevertheless, it is inconceivable that any increment could be advantageous.

5.38 In conclusion, it is possible to summarise (see Table 5.10) the contribution of genetic influence to disease by considering together the contributions listed in Tables 5.1, 5.4, 5.6 and 5.8. These figures can usefully be compared with those from population surveys of genetic or part-genetic disorders conducted in defined populations in the British Isles and in British Columbia. The first, published in 1959, pre-dates chromosome studies in man, while the second relates to children born in 1952–1983 who were identified through surveillance for congenital abnormalities. Although the data in Tables 5.10 and 5.11 have been compiled in different ways, there is a degree of similarity of trends between them. The relative lack of chromosomal cases in the Canadian survey reflects the mode of ascertainment. The same is true for the paucity of dominants, due to exclusion of those with late manifestation. The method of ascertainment also is likely to miss practically all sex-chromosome abnormalities because phenotype changes of these chromosome abnormalities might not be apparent in early life, or indeed can escape notice altogether. It is also likely that this survey includes trivial, as well as more serious congenital malformations. However, the data in Table 5.8 (from which those in Table 5.10 are a derived proportion) and those in Table 5.11 agree quite well in respect of frequencies of congenital malformations.

e. Mutation rates in man

5.39 The calculation of gene mutation rates in man is particularly difficult because it has to be based on a number of parameters, measurements of which are often difficult to obtain and frequently are no more than approximations. One source of difficulty is in understanding the nature of the mutations at the molecular level. The detailed knowledge now becoming available means that, in some cases, it is possible to be more precise about the type and origin of the mutational processes. Amongst other difficulties are the detection of rare phenotypes, the existence of genetic (as well as phenotypic) heterogeneity and the necessity of estimating the fitness of the mutant phenotypes. For these reasons, the most reliable methods are those which rely on the detection of a clear phenotype in a specific population and on family studies of those who have and who have not directly inherited the defect. This direct approach can be applied only to dominant conditions, and clearly is reliable only when the penetrance of the condition is complete, its expression in the individual is full and relatively invariate and, when non-genetic (phenocopies) and genetic heterogeneity do not exist. Even then, there is generally no information about the intricate nature and the molecular aspect of the mutational process at the chromosomal and genic levels. Furthermore, there is a bias when selecting for study of the mutation rate of the detrimental dominant mutations in man. Unavoidably, the more common dominant conditions are selected so that it is probable that the mutation rate estimates are biased towards higher values. However, under ideal conditions, reliable estimates of mutation rates can be made and expressed on a per-specific-locus basis, per generation. But such ideal conditions are seldom met, so that calculations of human mutation rates tend to be made even more indirectly and depend in general on many approximations.

5.40 Difficulties are particularly obvious when one is dealing with autosomal recessive conditions. It should be stressed that complete neutrality in heterozygous carriers, ie complete recessivity of the more detrimental mutations which are responsible in the homozygous state for lethal conditions of severe diseases, for example phenylketonuria, is unlikely. However, the possible detrimental effect in the carriers (if detriment exits) may be quite small and therefore may be difficult and, in practice, impossible to detect. Nevertheless, because of the high frequency of carriers in the population, even small detriments will result in a relatively prominent loss of the corresponding detrimental genes. For example, a recessive disease with a frequency of one in 40,000 (carrier frequency one in 100), which reduces viability of the homozygous mutants by 10%, even if it impaired the reproductive fitness of the carrier by only one per thousand, would lead to greater loss of the detrimental genes from the heterozygous carriers than from those affected by the disease. Yet it would be impossible to detect the small loss of fitness of the heterozygotes. Naturally, equilibrium could be maintained in this case only if the mutation rate were proportionally higher when there is heterozygote disadvantage than when the heterozygotes are selectively neutral and the disadvantage resides only in the homozygotes mutant state. The corollary is that, an increased mutation rate for such a gene would introduce new individuals with a detriment and so would break the existing equilibrium between new mutation and elimination of the detrimental gene. In this view, recessive mutations whose presence can remain unobtrusive for generations are of no small interest, as they might contribute substantially, by force of numbers of carriers, to detrimental effects.

5.41 It should be remembered that in general, the maintenance of equilibrium frequencies of some detrimental genes requires that gene losses from the population be balanced by corresponding gains through mutation. In respect of recessives, because the losses of such genes from carriers are proportionally quite sizeable compared with the losses from the homozygote mutants, the influence of fitness of the heterozygotes on estimates of gene mutation rates is important. Therefore, because of the uncertainties of the effect of recessive detrimental genes in heterozygous carriers, which at times is advantageous to them (see paragraph 5.36), mutation rate estimates of recessive genes are likely to be very unreliable. Table 5.12 thus sets out the mutation rates for several autosomal dominant and sex-linked diseases as estimates only. In many instances, when more than one value is given by authors, the maximum and minimum estimates have been listed. Attempts have been made to calculate the per locus mutation rates for conditions which are clearly clinical and genetically heterogeneous. For example, this has been done for severe mental retardation (2.4×10^{-5} per locus: Morton, 1960) and for dominant deafness (4.7×10^{-5} per locus: Crow, 1961). Accepting the assumptions inherent in these estimates, they tend to fall in line with the values estimated more specifically. In the course of population and family studies on biochemical variation of a small number of specific enzymes in man, it has been possible also to put an upper limit to the estimate of mutation rate. For these loci, this has turned out to be 2.24×10^{-5} per gene per generation. It can be seen that most mutation rates have values of the order of one in 10^{-5} to one in 10^{-6} per gamete per generation, and clearly, these orders of frequencies also reflect the fact that the more common types of disorders have been preferentially studied. It has been calculated that the median value for a fairly large number of X-linked mutations in man is 1.6×10^{-7}. Attempts have also been made, on the basis of data derived from the detailed biochemical resolution of mutant proteins like haemoglobin to estimate the per codon mutation rate (specific base substitution in a codon) and values of the order of one in 10^{-8} have been obtained (Evans, 1988).

5.42 Numerical and structural chromosome abnormalities can be considered as dominant changes and calculation of their mutation rates, except for some of the structural chromosome mutations, is a relatively simple matter. The mutation rates for all chromosome abnormalities taken together and relative to survivors at birth differ from the gene (or point) mutation rates by about two to four orders of magnitude. The majority of numerical chromosome abnormalities are new mutations and are only exceptionally inherited. Mosaics represent mostly somatic mutations, but some are probably consequent on germinal mutation. Thus from Table 5.1. it can be concluded that numerical mutations, detectable at birth, have a mutation rate of almost two per thousand gametes per generation. The total mutation rates for the structural chromosome abnormalities, mostly centric (Robertsonian) fusions and translocations, is about 0.23 per thousand gametes per generation (Evans, 1988).

Table 5.1: *Chromosome abnormalities in live births, per thousand**

Numerical:				
	45,X	0.07		
Sex chromosome trisomies	47,XXX	0.54		
	47,XXY	0.61	1.64	
	47,XYY	0.49		
	Other sex chromosome anomalies (mostly mosaics)	0.96		4.14
Autosomal trisomies	47,+D	0.12		
	47,+E	0.19	1.45	
	47,+G	1.14		
	Triploidy	0.02		
Structural:				
	Balanced D/D centric fusions	0.79		
	Balanced D/G centric fusions	0.21	1.93	
	Balanced translocations: Inversions	0.93		2.21
	Unbalanced translocations, Inversions and deletions	0.28		
Unclassified:		0.51		
Total:		6.86	(1 in 146)	

* Bochkov *et al.*, 1974
Jacobs *et al.*, 1974
Sergovich *et al.*, 1969
Friedrich and Nielsen, 1973
Lubs and Ruddla, 1970
Hamerton *et al.*, 1975
Nielsan and Sillensen, 1975

Table 5.2: *The prevalence of chromosome abnormalities in spontaneous abortions, perinatal deaths and live births* (all per 1000)*

	Spontaneous abortions				
Type of anomaly	<12 weeks (N = 1498)	<18 weeks (N = 225)	<28 weeks (N = 941)	Perinatal deaths (N = 500)	Live births (N = 43558)
Tetraploidy	38.05	47.06	12.75	0	0
Triploidy	122.16	54.90	40.38	2.00	0.13
Autosomal trisomy	330.44	250.98	151.98	28.00	1.24
Monosomy X	93.46	156.86	72.26	2.00	0.046
Other	30.70	39.22	27.62	24.00	4.43
Total all anomalies	614.81	549.02	304.99	56.00	6.26

*Boué *et al.*, 1975
Creasey *et al.*, 1976
Evans, 1977
Lauritsan, 1976
Machin, 1974

Table 5.3: *A. Chromosome abnormalities among spontaneous abortions (per cent)**

Autosomal trisomy	45,X	Triploidy	Tetraploidy	Structural anomaly	Mosaicism	Double trisomy	Autosomal monosomy	Sex trisomy	Others
50.6	19.6	16.1	5.3	3.7	2.7	1.6	0.2	0.2	0.3

B. Specific autosomal trisomies as a percentage of all trisomic spontaneous abortions

Specific chromosomes involved	16	21,22	14,15	2,7,8 18	4,9,10 13	20	3,5,6,11 12,19	1,17
Incidence, %	30	20	7.5	5	2.5	1.5	0.5	Nil

* Creasy, 1977

Table 5.4: *Estimated incidence at birth of some relatively common and better known autosomal dominant diseases*

Condition	Frequency per thousand	References
Huntington disease	0.1–0.22	Caro, 1977; Quarrel *et al.*, 1988; Baird *et al.*, 1988
Neurofibromatosis	0.07–0.4	Verschuer, 1962; Baird *et al.*, 1988
Wilm tumour	0.10	Young & Miller, 1975
Myotonia dystrophica	0.12	Grimm *et al.*, 1975
Tuberous sclerosis	0.03–0.06	Hunt & Lindenbaum, 1984
Retinoblastoma	0.055–0.066	Vogel, 1961; Saunders *et al.*, 1988
Achondroplasia	0.015–0.10	Rimoin, 1975
Diaphyseal aclasis (multiple exostoses)	0.05–0.1	Murken, 1963a; Murken, 1963b
Marfan disease	0.1	Pyeritz & McKusick, 1979
Familial adenomatous polyposis	0.083	Watne, 1982
Bilateral polycystic kidney (adult type)	0.8	Dalgaard, 1957
Profound childhood deafness	0.07–0.12	Nance & McConnell, 1973; Fraser, 1976
Waardenburg syndrome	0.0005–0.025	Fraser, 1976
Hereditary spherocytosis	0.22	Morton *et al.*, 1962
Acute intermittent porphyria	0.015–0.024	Stanbury, Wyngaarden and Fredrickson, 1972; Tschudy, 1973
Porphyria variegata	0.01	
Craniofacial dyostosis	0.04	Cohen, 1986
Others	?0.2	Bodmer and Cavalli-Sforza, 1976
Total	2.08–2.79	

Table 5.5: *Estimated incidence at birth of some of the more common or better known autosomal recessive disorders*

Condition	Frequency per thousand	References
Cystic fibrosis	0.4–0.5	Bodmer & Cavalli-Sforza, 1976 Nevin, Nevin & Redmond, 1983
Severe mental retardation (excluding PKU and other inborn errors of metabolism listed below)	0.8	Bodmer & Cavalli-Sforza, 1976
Phenylketonuria	0.09	Harris, 1975; Levy, 1973
Galactosaemia	0.016	Levy, 1980
Other aminoacidurias	0.28	Harris, 1975; Levy, 1973
Mucopolysaccharidoses	0.01–0.043	McKusick, 1972; Baird *et al.*, 1988
Tay-Sachs disease	0.001–0.003	Myrianthopoulous and Aronson, 1966
Severe childhood deafness	0.22–0.37	Cavalli-Sforza & Bodmer, 1971 Nance & McConnell, 1973
Childhood blindness	0.1	Cavalli-Sforza & Bodmer, 1971
Acute spinal muscular atrophy (Wernig-Hoffman)	0.039	Pearn, 1973; Pearn & Wilson, 1973
Friedreich's ataxia	0.01	Skre, 1980
Spinocerebellar ataxia	0.018	Skre, 1980
Cerebellar ataxia	0.012	Skre, 1980
Hereditary spastic paraplegia	0.019	Skre, 1980
Oculo-cutaneous albinism	0.04–0.1	Witkop, 1971
Ataxia telangiectasia	0.02	Sedgewick & Boder, 1972
Adrenogenital syndromes	0.1	Bodmer & Cavalli-Sforza, 1976
Others	?0.1	Bodmer & Cavalli-Sforza, 1976
Total	2.27–2.62	

Table 5.6: *Some genetic diseases that show marked regional variation*

Condition	Frequency per thousand	Region	References
Porphyria variegata	300 1.0	South African whites Caucasians generally	Diamond, 1987
Myotonia dystrophia	90 5.5	New Zealand Germany	Brock, 1972 Grimm, 1975
Huntington Chorea	17 8.4 3.0	Tasmania Canada Japan	Brock, 1972 Shokeir, 1975 Kishimoto, Nakamura and Sotokawa, 1959
Adrenogenital syndromes	200 20 2.5	Yupik Eskimos, Alaska Switzerland North America	Crosby, 1987 New and Levine, 1973 Polani, 1973
Cystic Fibrosis	40.0–50.0	North Europeans	Polani, 1973; Bodmer and Cavalli-Sforza, 1976
		Victoria, Canada	Brock, 1972
	1.0	Afro-Americans and Orientals (Chinese)	Bodmer and Cavalli-Sforza, 1976
		Hawaiians (non-Caucasoid)	Wright and Morton, 1968
Tay-Sachs disease	17.0–40.0 0.1–0.3	Ashkenazi-Jews Gentiles, Sephardi Jews	Harris, 1975 Harris, 1975; Polani, 1973; Myrianthopoulos and Aronson, 1966; Brock, 1972
Gaucher disease (adult type)	5.0–50	Ashkenazi Jews—Israel	Stanbury, Wyngaarden and Fredrickson, 1972; Brock, 1972
	'seems very rare'	Other Caucasians	Stanbury, Wyngaarden and Fredrickson, 1972
Hereditary tyrosinaemia	15.0–18.0	Chicontim Region of North East Quebec, Canada	Brock, 1972
	0.4	North East USA and East Canada	Polani, 1973
	0.07	Caucasians generally	Levy, 1973
Phenylketonuria (PKU)	16.7–22.2 8.5 'virtually absent'	Scotland, Ireland Caucasians generally Ashkenazi Jews	Levy, 1973
Cystinuria (I and II)	96.0 2.5	Sweden England	Bostrom and Hambraeus, 1964 Knox, 1958
Duchenne muscular dystrophy	41.0 21.2	Western Australia Caucasians generally	Brooks and Emery, 1977
Thalassaemia	1000	Mediterranean peoples, etc	Polani, 1973
Sickle cell anaemia	1000–2000	Africans	Polani, 1973

Table 5.7: *Estimated incidence at birth in males of some of the commoner sex-linked diseases*

Condition	Approximate frequencies per thousand *males*	References
Duchenne muscular dystrophy	0.25	Moser, 1984
Becker muscular dystrophy	0.01	Stevenson & Kerr, 1967
Haemophilia A	0.10–0.12	Stevenson & Kerr, 1967
Haemophilia B	0.03	Giannelli *et al.*, 1983
Icthyosis	0.20	Stevenson & Kerr, 1967
Mental retardation, fragile X (Martin-Bell)	1.0–2.0	Turner *et al.*, 1986
Mental retardation, non-fragile X	0.9	Herbst & Miller, 1980 Opitz & Sutherland, 1984
Childhood deafness	0.3–0.9	Stevenson & Kerr, 1967
Childhood blindness	0.02	Bodmer & Cavalli-Sforza, 1976
Lesch-Nyhan syndrome	0.1	Kelley & Wyngaarden, 1983
Others	?0.1–0.3	
Total	3.01–4.83	

Table 5.8: *Frequencies in the population (mostly in England and Wales) of the commoner abnormalities of development expressed per 1,000 births**

Condition	Frequency per 1,000 births	References
Anencephaly with or without spina bifida	1.11	OPCS, 1983
Spina bifida cystica without anencephaly	1.66	OPCS, 1983
Microcephalus	0.13	OPCS, 1983
Abnormalities of heart and great vessels	6.8–8.0	Bound & Logan, 1977; Campbell, 1973
Congenital hydrocephalus (without spina bifida)	0.74–1.4	Adams, Johnston & Nevin, 1982
Hypertrophic pyloric stenosis	2.2	Polani, 1973
Idiopathic equinovarus	3.77	OPCS, 1983
Congenital dislocation of the hip	0.9–3.0	Leck, 1974; Nevin, 1979
Cleft lip with or without cleft palate	1.0–1.4	Leck, 1974; Nevin, 1979
Cleft palate only	0.4	Polani, 1973
Hirschsprung's disease	0.2	Passarge, 1983
Inguinal hernia	10.0	Polani, 1973
Hypospadias	1.45	OPCS, 1983
Poly- and syn-dactyly	1.43–1.8	Leck, 1974; OPCS, 1983
Skeletal reduction abnormalities	0.46	OPCS, 1983
Total	32.25–36.98	

* Excluding conditions like renal agenesis and other renal anomalies, rectal and other intestinal atresias.

Table 5.9: *Relatively common conditions in which genetic factors play a variable role**

Condition	Approximate population frequency (per thousand)
Schizophrenia	8–10
Affective psychoses	4
Epilepsy	5
Diabetes mellitus	3–10
Total	20–29

* Polani, 1973

Table 5.10: *The overall influence of genetic factors on human disease*

Condition	Frequency per thousand	
Chromosome abnormalities	6.86	Table 5.1
Defects caused by single genes of major effects:		
Autosomal dominant	2.08–2.79	Table 5.4
Autosomal recessive	2.27–2.62	Table 5.5
X-linked	3.01–4.83	Table 5.7
Sub-total	14.22–17.10	
Congenital malformations*	16.12–18.49	Table 5.8
Complex disorders**	7.00–10.00	Table 5.9
Other diseases	?	
Total genetic contribution (approximately)	37.34–46.59	

* Conditions listed in Table 5.8; genetic contribution, say half

** Conditions listed in Table 5.9; genetic contribution, say one-third

Table 5.11: *Frequencies of genetic and part-genetic disorders in two population studies, per 1,000 livebirths*

Condition	Stevenson, 1959	Baird *et al.*, 1988
Autosomal dominant	33.2*	1.4
Autosomal recessive	2.1	1.7
X-linked	0.4	0.5
Chromosomal abnormalities	–	1.9
Congenital malformations	14.1	23.1
Other multifactorial disorders	14.8	23.9
Unknown (genetic)	–	1.2

* Half of the anomalies included are of trivial import, and there are conditions not now considered autosomal dominants.

Table 5.12: *Mutation rates for some genetic defects*

Condition	Mutation rate per gamete per generation	References
Autosomal Dominant Mutations:		
Huntington disease	1.5×10^{-6}	Becker, 1966
Neurofibromatosis	$\sim 0.8 \times 10^{-4}$	Evans, 1988
Wilm tumour	$\sim 0.35 \times 10^{-4}$	Evans, 1988
Myotonia dystrophia	$0.5\text{–}1.3 \times 10^{-5}$	Harper, 1979
Tuberous sclerosis	$6\text{–}10 \times 10^{-6}$	Vogel and Rathenberg, 1975
Retinoblastoma	$\sim 0.25 \times 10^{-4}$	Evans, 1988
Achondroplasia	$3\text{–}9 \times 10^{-5}$	Connor *et al.*, 1985
Diaphyseal aclasis	$6\text{–}9 \times 10^{-6}$	Vogel and Rathenberg, 1975
Marfan disease	$4\text{–}6 \times 10^{-6}$	Lynas, 1956/57
Familial adenomatosis polyposis	$\sim 0.17 \times 10^{-4}$	Evans, 1988
Adult polycystic kidney	$65\text{–}120 \times 10^{-6}$	Vogel and Rathenberg, 1975
Hereditary spherocytosis	22×10^{-6}	Morton *et al.*, 1962
Acrocephalo-syndactyly	$3\text{–}4 \times 10^{-6}$	Blank, 1960
Nail-patella syndrome	2×10^{-6}	Renwick and Izatt, 1965
X-linked mutations:		
Duchenne muscular dystrophy	7×10^{-5}	Moser, 1974
Becker muscular dystrophy	$1\text{–}2 \times 10^{-6}$	Stevenson and Kerr, 1967
Haemophilia A	$32\text{–}57 \times 10^{-6}$	Vogel and Rathenberg, 1975
Haemophilia B	$2\text{–}3 \times 10^{-6}$	Vogel and Rathenberg, 1975
Ichthyosis	1×10^{-6}	Stevenson and Kerr, 1967
Testicular feminisation	5×10^{-6}	Jagiello and Atwell, 1962
Incontinentia pigmenti	$6\text{–}20 \times 10^{-6}$	Vogel and Rathenberg, 1975
Orofaciodigital syndrome	5×10^{-6}	Vogel and Rathenberg, 1975

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6. The monitoring of human populations for mutational changes

6.1 Accepting the overwhelming direct evidence that mutational changes in humans are responsible for a significant proportion of genetically determined disease states and congenital malformations, and the indirect evidence that exposure to mutagens can increase the incidence of such mutations, it is important to ask whether it is possible to monitor populations and the environment in which they live to ensure that individuals and groups of individuals are not being exposed to known or unknown mutagens.

6.2 The principle of monitoring for the presence of toxic agents or pathogens in the general environment, or in a variety of specialised environments associated with particular types of industry, has long been established, and is practised by Public Health Authorities or similar bodies. Monitoring in these contexts is carried out at two levels. Firstly, there is the continuous surveillance of the environment to detect the presence of physical, chemical or biological agents which can produce specific effects on individuals who are 'over-exposed' (ie on the coalminer's canary principle). Secondly, there is the surveillance of individuals who are known to be at risk of exposure, for example, to a particular toxin, to ensure the prevention of future exposure. The second is necessarily retrospective, detecting exposure after it has occurred, but enabling steps to be taken to prevent further exposure. In principle, similar monitoring for exposure to mutagens is possible but there are special problems.

6.3 Agents that are mutagenic may be identified individually or collectively by use of mutagen testing systems so that, once identified, monitoring by physical or chemical means is possible. This is particularly relevant in cases where individuals might be exposed to a chemical agent of known mutagenicity during the course of their work, so that the levels of this agent in the working environment may be continuously monitored to ensure that no over-exposure occurs. It has become apparent, however, that humans are inevitably exposed to a very large number of mutagens during everyday life. Some are present in plant material that we eat—they are often 'nature's pesticides' developed by plants for their own protection (Ames, 1983), others are generated by chemical reactions during cooking (Sugimura, *et al.*, 1986), others are endogenous, generated by the metabolic processes of our own bodies (Ames and Saul, 1988). A very small proportion indeed are man-made chemical pollutants. The contribution of mutagens from any of these different sources to the human mutation rate is quite unknown and this causes formidable problems both in the estimation of genetic risk from exposure to any particular man-made chemical, and in the estimation of possible effects of very low levels of mutagenic agents. At the present stage of knowledge, therefore, the prime aim must be to identify and attempt to minimise exposure to agents constituting a potentially large risk over and above that which is unavoidably associated with human life.

6.4 Because of the difficulties in determining which exposures to mutagens constitute significant risks, the question arises as to the feasibility of monitoring individuals or populations for evidence of mutagenic exposure. Most important is the fact that over-exposure to mutagens does not normally result in one specific and easily observed clinical manifestation as it does, for example, in the case of over-exposure to a bacterial toxin, a specific virus or metals such as lead and cadmium. The consequences of induced mutation may be expected to be manifest in later life and in a variety of ways in the descendants of exposed individuals. In considering the monitoring of humans, one may consider firstly, evidence of exposure, secondly, some sort of biological response in the exposed individual, and thirdly, evidence of mutation induction in the offspring of exposed individuals (germ line effects).

Evidence of human exposure

6.5 Evidence that people have been exposed to mutagens can be sought by examining body fluids or tissues for signs characteristic of mutagenic activity. A variety of methods for doing this are under investigation (Berlin *et al.*, 1984). These include indirect assays, for example, short- term tests for determining the mutagenic activity of body fluids (Venitt *et al.*, 1986a; Venitt, 1987), and direct assays, for example, detecting adducts in DNA extracted from cells or tissues (Venitt *et al.*, 1986b; Perera, 1987). At present, these methods are experimental rather than routine, and only further development and validation will allow us to decide which (if any) of these methods will be suitable for routine monitoring.

Tissues and body fluids available for monitoring

6.6 Monitoring human populations depends on a supply of samples of body fluids or tissues in amounts sufficient to allow reliable assay by the chosen method. Materials which meet this requirement and which comply with ethical and practical constraints are saliva, sputum, blood, urine, faeces and semen. Other materials which are easy to collect include hair, nail-clippings, sweat and cells from the buccal mucosa. Body fluids and tissues such as skin, gastric juice, cervical mucus, bile and peritoneal fluid require invasive techniques for collection and are not suitable for monitoring human populations.

Methods of assay

6.7 Table 6.1 shows some examples of assays currently under investigation. The presence of a mutagen or its metabolite in a body fluid or tissue, or the demonstration of mutagenic activity of a body fluid in a bacterial assay is an indicator of absorption or internal dose, rather than of a biological effect on the exposed individual. Chemical or physical methods of detecting the presence of mutagens or their metabolites usually depend on prior knowledge of the nature of the putative exposure. For example, immunoassay of urinary aflatoxin is based on the availability of immunochemical markers of high specificity to that compound. The detection of excised adducts in urine resulting from the repair of mutagen-modified DNA suffers from the same limitation. On the other hand, urinary mutagenicity assays are more eclectic since the identity or type of putative mutagen does not need to be known in advance in order to perform the assay and the same end-point can be used to detect a variety of different substances in circumstances where there might be a mixed exposure.

6.8 Detecting the presence of adducts in DNA from tissues or cells from exposed individuals is a more precise measure of internal dose, and also provides evidence that a mutagenic substance has reached and reacted with a critical target. Immunoassays, in which polyclonal or monoclonal antibodies to modified DNA or specific DNA adducts recognise and bind to the adducts of interest, are exquisitely sensitive and can quantitatively detect as little as one adduct per 100 million DNA nucleotides (Perera, 1987). In addition to assaying DNA from tissues or cells for the presence of adducts, immunoassay can also be used for detecting the presence of excised DNA adducts in urine of exposed individuals (Perera, 1987). However, prior knowledge of the nature of the adducts is required in order to produce the appropriate reagents for the assay.

6.9 The technique of 'post-labelling' goes a long way to avoid this problem. In this type of assay, tiny amounts of DNA are broken down to individual deoxynucleotide monophosphates using appropriate enzymes. The mixture of deoxynucleotides is then incubated with ^{32}P-ATP in the presence of phosphorylating enzymes, producing ^{32}P-labelled deoxynucleoside diphosphates of extremely high specific radioactivity. The ^{32}P tag serves as a very sensitive marker in the subsequent chromatography which separates normal deoxynucleotides from those which have been modified by adduct-formation. This technique is extremely sensitive (one adduct per 10^9 or 10^{10} DNA nucleotides), especially to bulky aromatic adducts (Perera, 1987).

6.10 Fluorescence spectrometry is a direct chemical method for detecting DNA adducts. In its present state of development it is not as sensitive as immunoassay or post-labelling (Venitt *et al.*, 1986b).

6.11 The detection of alkylated amino acid residues in haemoglobin using physical techniques such as mass-spectrometry has been developed as a surrogate for the technically demanding methods required for DNA-adduct detection. The method is very sensitive (it can detect one nmol of hydroxethylhistidine per gramme haemoglobin in blood from a finger-prick). However, its suitability for monitoring human exposure to mutagens other than alkylating agents remains in doubt.

Confounding variables in monitoring human populations

6.12 One of the greatest problems in biological monitoring is incomplete knowledge of the normal 'background' levels of, for example, urinary mutagenicity or DNA adducts, and the influence of life-style habits on this background. The two most serious confounding variables are likely to be tobacco and diet. Other possible causes of bias are intake of drugs, intoxicants and medicines, and disease states (Berlin *et al.*, 1984; Venitt *et al.*, 1986a; Venitt, 1987). The principles underlying the use of the extremely sensitive methods described above for human monitoring are similar to those which govern epidemiological studies. In most cases, donors will be drawn from outbred human communities. Each donor will be genetically unique; each donor creates his or her own *milleu interieur* both by personal choice (type and amount of diet; to smoke or not to smoke; to consume alcohol or to abstain) and by conditions imposed, for example, by passive smoking, occupation, ill-health or living conditions. Clearly, whatever the purpose of the study, the investigator will find it difficult or impossible to select groups of donors sharing identical life-styles, and will therefore have to accept that the selection of donors will present problems and that great care must be taken to avoid introduction of bias.

Evidence of biological response in humans

6.13 Another approach to monitoring populations for exposure to genotoxic agents is to determine mutagenic changes in somatic cells and in sperm samples from individuals known to be at risk (see Table 6.2). The response of certain strains of rodents to mutagens can be detected by an increase in the incidence of morphologically abnormal sperm although this does not necessarily reflect a genotoxic effect. The background incidence of such sperm in healthy fertile human males is quite high, so small increases due to exposure to mutagens are difficult to detect. Nevertheless, this approach has had some success in man. The chromosome constitution of human sperm can be revealed by *in vitro* fertilisation of hamster eggs (Rudak *et al.*, 1978; Martin *et al.*, 1983; Brandriff *et al.*, 1984). In principle, this provides a means for monitoring both structural and numerical chromosomal changes, but the technique is rather demanding and has not been used as a routine procedure. It has, however, been successfully employed to demonstrate the occurrence of chromosome aberrations in the sperm of patients whose testes had been irradiated (Martin *et al.*, 1986). Direct monitoring of chromosomal changes in human eggs is not currently possible.

6.14 Methods for the detection of gene mutations in somatic cells by searching for altered, defective, or missing gene products are now available, by selecting in a medium where only the mutant cells will grow. Growth of the cells can be estimated visually or by measurement of incorporated radiolabel in the growing cells. The method is currently being explored with peripheral human lymphocytes. It is clear that the frequency of at least one type of gene mutation is influenced by factors other than exposure to mutagens and the use of the method in the latter context must be approached with caution. The examination of red blood cells for altered gene products due to mutations in progenitor cells is also feasible in principle, but it requires expensive and sophisticated equipment and has been successfully accomplished in only a few laboratories.

6.15 Recent developments in human molecular genetics have led to the emergence of a very wide range of recombinant DNA probes that are specific for particular chromosomes and chromosome regions. There have also been significant improvements in the techniques of *in situ* hybridisation to reveal sites of probe hybridisations on chromosome and nuclei. These techniques are beginning to be used to detect the occurrence of abnormalities in chromosome numbers in human somatic, including embryonic, cells and in spermatozoa. They provide a means to assay for the induction and occurrence of aneuploidy (Cremer, *et al.,* 1986; West *et al.,* 1987).

6.16 Currently, the best established approach for human monitoring involves the examination of peripheral lymphocytes for chromosome aberrations. Another useful method involves the identification of sister chromatid exchange (SCE). Scoring chromosome aberrations is being successfully used to monitor exposure in individuals who might be exposed to ionising radiation. Both chromosome aberrations and SCE are being utilised to monitor *groups* who may be exposed to known chemical mutagens in the course of their work and they might well be applicable eventually to studies of *individuals* as is the case for radiation (Carrano and Natarajan, 1988).

6.17 If preliminary studies have indicated the possibility of unusually high exposure of a group of people to a mutagen, it might be possible to survey for fetal loss or congenital abnormalities. Large groups would need to be considered, however, and there could be difficulty in identifying appropriate control populations. Nevertheless, such studies would be of great value in the calibration of human response to exposure to mutagens.

Evidence of germ line mutation in humans

6.18 Changes in the rate of germ line mutation in humans can at present be studied only in populations and not in individuals and their offspring. Even so, the search for germinal effects of putative mutagens is fraught with difficulties created by the limited number of suitable genetic end-points, the rarity of any particular gene mutations, and the need for large numbers of 'exposed' and 'unexposed' individuals if statistically usable results are to be obtained. Because of these difficulties it has not yet been possible to demonstrate convincingly the induction of heritable mutations in humans by any agent. The potentialities and problems of mutation epidemiology have been comprehensively set out in the report of an ICPEMC Committee (Miller, 1983a) and its associated working papers (eg Miller, 1983b).

6.19 Numerical and structural chromosomal anomalies contribute heavily to the burden of human genetic disease, and since most of them arise *de novo* they would seem to offer great potential for use in mutation epidemiology.

However, it has not so far been possible to demonstrate convincingly an effect of environmental mutagens on the incidence of such disorders, although there are differences in incidence between different populations. The commonest chromosomal disorder is Down syndrome and a recent meeting concluded that: there was no significant evidence indicating an upward trend in the live-birth incidence of Down syndrome, even after correction for induced termination and patterns of child bearing; there was no evidence for consistent seasonal fluctuation in Down syndrome; there was no clear evidence for any single aetiological factor, or combination of factors, responsible for non-disjunction resulting in Down syndrome; and in view of the complexities involved in the aetiology of this condition (which is heavily influenced by maternal age, by non-disjunction occurring at different frequencies in different divisions, by the loss of trisomy 21 fetuses due to abortions, and by the interaction of two or more of these factors), this particular syndrome did not readily conform to the requirements that would be necessary if it were to be used as a general end-point in epidemiological studies of mutation in man (Evans *et al.,* 1986). For monitoring populations, the two approaches most likely to be useful are (i) the examination of chromosome abnormalities in foetuses diagnosed prenatally at amniocentesis and (ii) the examination of all cases with Down syndrome and Patau syndrome for the proportion of mutant interchange trisomies.

6.20 In considering genetic disease due to gene mutations, consideration has been given to surveillance of so-called 'sentinel phenotypes'. A sentinel phenotype is a clinical disorder that occurs sporadically as a consequence of a single, highly penetrant mutant gene, is a dominant or X-linked trait of considerable frequency and low fitness, and is uniformly expressed and accurately diagnosable with minimal effort at or near birth. The number of possible sentinel phenotypes is around 50. As markers of human mutations, sentinel phenotypes have the advantage that they confer significant health problems. Unfortunately, there are severe disadvantages that have so far prevented the launching of a sufficiently large study of the value of these phenotypes in mutation epidemiology, although a pilot study is underway in Hungary (Mulvihill and Czeizel, 1983).

6.21 About 15% of recognised pregnancies abort spontaneously or terminate in a still birth and the loss prior to recognition of pregnancy is probably greater still. Chromosome mutations are known to be responsible for many spontaneous abortions but there are severe problems both in ascertainment and in eliminating non-genetic causes. A similar array of difficulties attends the use of congenital disorders as indicators of mutagenic events.

6.22 The problems associated with the surveillance of human populations for inherited disorders due to newly arising gene mutations has prompted a search for molecular methods that will reveal mutations without involving diagnosis. As early as 1973, Neel, Tiffany and Anderson suggested that mutational changes could be monitored by screening plasma for the products of some twenty genetic loci using one-dimensional electrophoresis to separate proteins of different electrical charges. This has been applied to populations in the

USA, Japan, South America, the UK and the USSR. The number of loci screened is now approaching a million and the human mutation rate appears to be around 1.5×10^{-5} per gene per generation. The one-dimensional method is restricted to very large populations (and is accordingly very expensive) because it is possible to analyse only two or three dozen proteins per blood sample. More recently, two-dimensional separation, first using isoelectric focussing to separate on the basis of charge, and then using electrophoresis to separate on the basis of size, has been proposed (Anderson and Anderson, 1977). Using this approach, it should be possible to monitor the protein products of up to 100 genetic loci in plasma, and possibly proteins of up to 200 loci in blood cells. With digital imaging, up to 1000 proteins could be distinguished on one gel. So far this method has not been widely applied.

6.23 Protein variant methods could soon be overtaken by methods looking directly at DNA sequence damages, using techniques being developed in the field of DNA technology. A recent meeting (Delehanty *et al.*, 1986) identified and analysed six DNA methods to defect human heritable mutation, including several methods created at the meeting. Although all these methods will require considerable development and none are remotely ready for field application, it is likely that refinements in DNA experimentation will soon permit the analysis of mutation in human populations.

Table 6.1: *Examples of methods for monitoring exposure of human populations to mutagens*

End-point used in assay	Method of assay	Examples of material
Presence of mutagen or its metabolite	Physical and chemical immunoassay	Urine, blood, faeces, sweat
Mutagenicity	Bacterial mutation assays	Urine, blood, faeces, sweat
DNA adducts	Immunoassay, post-labelling, fluorescence spectrometry	White blood cells, buccal mucosal cells, exfoliated bladder cells, sperm
Protein adducts	Mass-spectrometry, amino acid analysis, high-performance liquid chromatography, gas chromatography	Red blood cells
Excised adducts	High-performance liquid chromatography, fluorescence	Urine

Table 6.2: *Examples of assays for monitoring genetic effects in the cells of individuals exposed to mutagens*

Tissue sampled	Cells examined	End point
Peripheral blood	Lymphocytes	Chromosome aberrations, Sister chromatid exchanges, Micronuclei, Mutants resistant to 6-thioguanine
	Red blood cells	Glycophorin mutants, Haemoglobin mutants
Seminal fluid	Spermatozoa	Morphological abnormality, Chromosome aberrations
	Spermatocytes	Micronuclei

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7. Recommended test systems for mutagenicity and pre-screening for carcinogenicity

Introduction

7.1 It is clear from the preceding chapters that the mutagenic properties of a chemical present a potential health hazard due both to the possibility of inherited disorders and to the induction of malignancies. It is also apparent that information on the three levels of mutation, namely gene, chromosome and aneuploidy, is necessary to provide a comprehensive coverage of the mutagenic potential of a substance. This would also appear to be true when assessing carcinogenic potential, since all three types of mutation have been shown to be associated with the activation and expression of oncogenes and loss or inactivation of tumour suppressor genes. At present, no single validated test can provide information on all these three end-points. There is thus a need to subject each substance to a series of tests. There can also be alterations to DNA involved in carcinogenesis that are distinct from end-points which depend on mutagenicity, for example, in aberrant gene regulation (where a gene is not turned off). In addition, there are genetically relevant targets other than DNA, for example, components of the mitotic spindle, disruption of which can produce aneuploidy.

7.2 It is the objective of this chapter to consider the tests involved, and to set out a testing strategy comprising those methods which are believed to be the most informative and best validated and hence likely to be the most helpful in a legislative context. Since a very extensive literature exists on the various test procedures and the important practical aspects of such studies, a description of methodologies is not provided and readers are referred to an extensive bibliography at the end of the guidelines.

7.3 The choice of appropriate assays is made difficult by the very large number of mutagenicity assays discussed in the scientific literature. For example, the EPA Gene Tox Data base now contains information on over 70 different assays. The tests employ a wide variety of organisms, including bacteria, yeasts and other eukaryotic microorganisms, and mammalian cells studied *in vitro*, as well as whole mammals where effects in either somatic or germ cells can be measured. A number of different genetic end-points are available. The numerous assays may be divided on the basis of these end-points measured (eg gene mutation, chromosome aberrations, 'indicator' tests for DNA damage) and on consideration of the different phylogenetic levels represented. However, many of the assays overlap in terms of the specific end-point or organisms, and it is clearly necessary to concentrate on a few studies that have been shown to have the most practical value in this area.

A strategy for testing for mutagenicity

7.4 It is recommended that the screening studies should investigate the mutagenic potential of the substance using *in vitro* tests. Few compounds are uniquely positive *in vivo* and provided that, for example, different concentrations of the exogenous metabolic activation system are investigated and adequate protocols are employed, almost all *in vivo* mutagens would be expected to have demonstrable activity *in vitro*.

7.5 Furthermore, it is appropriate to concentrate on a relatively small number of assays, using sensitive methods particularly chosen to avoid false negatives. In this regard, the assays should be designed to provide the most likely chance of detecting potential activity, with respect to (a) the exogenous metabolic activation system; (b) the ability of the compound or its metabolite(s) to reach the target DNA; (c) the ability of the genetic test system to detect the known variety of mutational event. The assays should be carried out to the best available protocols in the literature.

7.6 In addition it is important that all *in vitro* screening tests are repeated in a further independent experiment; the objective of this is to confirm the initial result. It is not necessary to carry out the study in an identical fashion to the initial experiment. Indeed, in some cases it might be preferable to alter certain aspects of the study (eg concentration levels investigated) so as to obtain more useful data.

7.7 There may be instances where alternative tests to those specified are more appropriate, and it is important that a flexible approach is adopted. Each compound should be considered on a case-by-case basis with regard to the selection of tests, as well as to the interpretation of results.

Stage 1: *In vitro* tests

7.8 The most widely used *in vitro* assay is the Salmonella reverse mutation assay for gene mutations using the strains of *Salmonella typhimurium* developed by Ames and his colleagues. The very extensive data base available for this assay justifies its inclusion in any initial testing package. Strains of bacteria capable of detecting both base-pair and frame-shift mutations must be included, the best validated strains being TA98, TA100, TA1535, TA1537 and TA1538. Other strains of Salmonella and also of *Escherichia coli* may be appropriate in certain circumstances. Testing should be carried out both in the presence and absence of an exogenous metabolic activation system.

7.9 The Salmonella assay, whilst being an efficient primary screen for detecting compounds with inherent potential for producing gene mutations, does not detect all compounds with mutagenic potential and additional *in vitro* assay(s) are necessary.

7.10 The Committee thus recommends that, in the initial testing package (Stage 1), a second assay should be performed to complement the Salmonella assay.

7.11 The second assay should employ a different end-point, namely the production of chromosomal damage, and should use mammalian cells, either cell lines or lymphocyte cultures. An *in vitro* cytogenetic assay for chromosome aberrations using metaphase analysis is at present the most appropriate assay. The rationale for this includes the observation that certain carcinogens (eg Actinomycin D, inorganic arsenic compounds) produce chromosome aberrations in such assays but give negative results in the Salmonella assay. Other groups, eg the International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC), have, on the basis of the results from collaborative studies, proposed that an *in vitro* chromosome assay may be a useful complement to the Salmonella assay. Furthermore, the assay has been used extensively for many years and its value is well established. Nevertheless, other end-points for clastogenic damage can be suitable if adequately validated.

7.12 A combination of these two assays will not detect a small proportion of agents with the potential for *in vitro* mutagenicity. For substances likely to be widely distributed, or to which significant human exposure is expected or intended, a further mammalian cell assay is considered desirable. This should employ a different cell type from that used for the cytogenetic assay (since this will increase the range of metabolic capabilities) and it should detect the induction of gene mutations (since a small proportion of agents can specifically or preferentially induce this type of change). Some mammalian cell gene mutation protocols that have been widely employed, particularly some of those involving the use of Chinese hamster cells, are now considered to be insufficiently sensitive on purely statistical grounds relating to the size of the population that is examined. Of the available systems, the TK locus in L5178Y mouse lymphoma cells has the advantage of detecting not only gene mutations but also various sizes of chromosome deletions.

7.13 There is growing evidence that spurious 'stress-related' positive results, which do not reflect intrinsic mutagenicity, are occasionally seen in mammalian cell assays (Brusick, 1987). These effects can, for example, occur with exposures that involve low pH or high osmolarity. Such positive results, attributable to pH effects, have also been reported in mutation assays in bacteria (Musarrat and Ahmad, 1988). These effects are not well characterised or understood, but their existence needs to be recognised and their relevance considered on a case-by-case basis.

7.14 All mutagenicity studies should be carried out to the most rigorous protocols. Provided that this is done, there is little to be gained from further *in vitro* testing. It is then necessary to investigate whether any intrinsic mutagenic properties of the compound detected *in vitro* can be expressed *in vivo* in mammals (ie Stage 2). Furthermore, information on the absorption, metabolism and distribution of the compound in mammals will provide additional, pertinent data to enable an assessment of hazard to be made.

7.15 The *in vitro* studies described above will not provide any information on interaction with the spindle apparatus or the induction of aneuploidy. Preliminary evidence for the ability of a compound to interact with the mitotic spindle apparatus can be obtained by examination of the effect of the test agent upon mitosis of treated cells in culture and harvesting without colchicine treatment. Evaluations of mitotic index and metaphase frequency provide evidence for such effects. More detailed information may be obtained by the use of appropriate staining procedures to highlight both chromosome and spindle structure. The ability of a chemical to induce chromosome aneuploidy may be assayed by some modification of the *in vitro* metaphase assay. In such investigations, it is necessary to use cell lines with a stable chromosome number and modification of hypotonic treatment might be necessary to reduce artifactual changes in chromosome number. The need for such studies in the evaluation of the mutagenic properties of a chemical should be considered on a case-by-case basis.

7.16 None of these studies, however, will provide information on gene recombination events. Data on both mitotic crossing-over (the exchange of segments of DNA between genes or between a gene and its centromere) and mitotic gene conversion (transfer of segments of DNA within a gene) can be provided by studies in the yeast *Saccharomyces cerevisiae*. These effects, which are regarded as indicators of DNA damage, may provide useful supplementary data in certain instances.

Stage 2: *In vivo* tests

7.17 *In vivo* studies provide invaluable data on the ability of a compound to express mutagenic properties in the whole animal. There are numerous reasons why activity shown *in vitro* might not be observed *in vivo* (for example, lack of absorption, inability of the active metabolite to reach DNA in target cell, rapid detoxification etc). *In vivo* data are therefore essential before any definite conclusions can be drawn about a given substance being potentially hazardous. The results of *in vivo* studies have thus a unique importance in hazard assessment.

7.18 *In vivo* tests can also detect substances that act only *in vivo*, although experience has shown that these are extremely uncommon. Provided that the three *in vitro* studies recommended in Stage 1 are carried out to comprehensive protocols, it is felt that practically all compounds with mutagenic potential will be detected. Thus it is not justifiable to use *in vivo* methods for general screening purposes when adequate alternatives exist which do not use live animals. However, in instances where a substance is designed to be administered to humans at high dose levels, or where moderate but prolonged levels of human exposure are expected, it is considered prudent, for additional reassurance, to have data from an *in vivo* assay.

7.19 The objective of *in vivo* studies is to show whether the substance has the ability to express activity *in vivo* and the route of administration should be chosen accordingly. Routes of exposure that are unlikely to give rise to significant absorption in the test animal should be avoided. Thus, if it is known that the compound is poorly absorbed by the oral route, the intra-peritoneal route should be used; if it is poorly absorbed through the skin, but fairly well absorbed orally, the latter route would be acceptable. There may be a case for a follow-up study using a route of administration that reflects exposure conditions likely to occur in use at a later stage.

7.20 By far the largest data base for *in vivo* mutagenic activity is provided by the assays for chromosome damage in bone marrow. Such damage may be determined either by a conventional metaphase analysis study, usually in rats, although other species may be used (and can be more appropriate in specific cases). Alternatively, the rodent micronucleus assay may be used which indirectly detects chromosome damage by measuring micronuclei in newly formed cells in bone marrow. This assay, unlike the metaphase analysis, will also detect spindle inhibitors. The micronucleus assay is somewhat easier and cheaper than the metaphase analysis study. It may be used to identify the induction of both structural and numerical chromosome aberrations. Micronuclei containing whole chromosomes (as opposed to fragments) may be presumptively identified by their size. More definite identification of micronuclei containing whole chromosomes will be dependent upon technical developments, such as the use of specific antibodies for the identification of centromeres.

7.21 Although such studies demonstrate activity *in vivo* in cells of the bone marrow, this tissue is readily accessible to compounds in the blood, and a large proportion of *in vitro* mutagens (and compounds that are known carcinogens in man) are active in these assays.

7.22 It is recommended that compounds investigated *in vivo* should be examined using either the bone marrow metaphase assay, or the micronucleus test.

7.23 However, as with the *in vitro* screens, there is a need for an assay that can complement the *in vivo* bone marrow assay by using tissue other than the bone marrow. Such an assay will be needed only if there is clear, unequivocal evidence for mutagenicity in one or more of the *in vitro* tests, and if the *in vivo* assay for chromosome damage in the bone marrow is negative. This is a difficult area, as, at present, no practical methods are widely available to screen for gene mutation *in vivo* in mammals.

7.24 The sex-linked recessive lethal assay in the fruit-fly *Drosophila melanogaster* measures gene mutations and small deletions *in vivo*, but the assay appears to be fairly insensitive, especially with certain classes of compound (for example, aromatic amines and azo dyes) and there is the difficulty of interpreting the significance of the results to mammals. It is not appropriate as an *in vivo* assay (and it has no place in a primary *in vitro* screen).

7.25 The dominant lethal assay, although it may have a place in confirming that effects seen in somatic cells also occur in germ cells, has relatively low sensitivity and fails to detect many somatic cell mutagens. It therefore is not recommended for use in initial screening.

7.26 In the mouse spot-test, pregnant mice are dosed, the target cells being the melanoblasts in the developing embryos. Special strains of mice have to be used so ensure that the developing embryos are heterozygous for a number of coat colour genes. A mutation in the dominant allele of such a gene results in the expression of the recessive phenotype in its descendant cells, constituting a spot of changed colour in the coat of the resulting mouse. The available results from a number of laboratories are fairly encouraging. However, the test has several disadvantages. Conflicting results have been obtained from different laboratories, and further inter-laboratory validation is desirable. The specific strains of mice needed are not widely available—at least 300 off-spring must be scored at each dose level—and considerable expertise is required to correctly score the spots. The method is little used in the UK.

7.27 An alternative approach, which requires far fewer animals, is to investigate unscheduled DNA synthesis (UDS) in rat liver *in vivo*. The end-point measured is indicative of DNA damage and may serve as a surrogate for genetic alterations *in vivo*. Initial results look encouraging, but problems remain regarding, for example, the selection of sampling times and duration of treatment. The overall duration of the study is also fairly long, as compared with, for example, the micronucleus test. Few laboratories have experience of the technique and further validation of this approach, using a wide range of compounds in several different laboratories, to give a better indication of the robustness of the assay, is urgently required.

7.28 Another method, which uses the liver as target organ, is based on the detection of micronuclei in the hepatocytes of treated animals. It is necessary to stimulate cell proliferation in the liver to allow the production of a clastogenic event to be expressed, and this presents some technical difficulty. The procedure is thus more complex than the bone marrow micronucleus test and less information is available at present on this approach than on the liver UDS assay.

7.29 In addition, for compounds that can be readily shown by biochemical or biophysical techniques to produce DNA damage in bacteria, or in cells in culture, it might be possible to investigate such effects *in vivo* in the whole animal. Techniques using radio-labelled material to detect covalent binding to DNA for example, are well established, and more recent methods that do not require such materials are becoming available.

7.30 There might also be a need for consideration of *in vivo* assays in other species in the case of the chromosome aberration assays, and also for the development of assays such as UDS at other sites to investigate local action in potential target tissues (stomach, skin).

7.31 In summary, it is recommended that, mutagenic activity observed *in vitro* is followed up by an *in vivo* investigation using a bone marrow assay for chromosome damage (metaphase analysis or the micronucleus test), to confirm that the mutagenic activity can be expressed *in vivo*. If negative results are obtained in this assay, it is desirable to investigate the compound in a second assay using tissue other than bone marrow. No recommendations can be made for a specific assay at this stage, as this will depend on several factors, including the nature and biological properties of the compound, its intended use, and the available expertise for mutagenicity testing. Data from adequate studies on absorption, distribution, metabolism and excretion will also be particularly valuable in this regard, in addition to the information from the *in vitro* mutagenicity assays. A flexible approach is necessary when considering the most appropriate additional *in vivo* study (or studies) in a given situation.

Stage 3: Germ cell assays

In vivo tests in the intact mammalian germ cell system

7.32 Because there is no good evidence for the existence of mutagens that induce mutations only in germ cells, it is considered that germ cell studies need not form a part of the screening package. However, since it is clear that not all somatic cell mutagens are germ cell mutagens, germ cell data might be called for in risk assessment studies of known or suspected mutagens. In this regard, a distinction should be made between tests that assay genetic damage only in germ cells and those that provide quantitative evidence for the heritable component of induced gene or chromosomal damage. In addition, it is important to recognise that (i) the various stages of spermatogenesis in the male (spermatogonial stem cells, differentiated spermatogonia, spermatocytes, spermatids and spermatozoa) can display quantitatively and qualitatively different responses even to potent mutagens and (ii) in females, differing responses can also be obtained with oocytes at different stages of maturation. As some of these tests are particularly demanding in terms of numbers of animals and/or experimenters' time, it is important to choose the most appropriate test, bearing in mind the germ cell stage under investigation.

7.33 When considering the need for germ cell data it is essential to take into account all the data available on the toxicological profile of the compound. Assessment of the pharmacokinetic and toxicity data (were morphological effects seen in the gonads?) can provide valuable evidence that the compounds reach the gonads.

7.34 The methods available are listed below:—

7.34.1 *In vivo cytogenetic studies to detect chromosome aberrations in differentiated spermatogonia.* Analyses at mitosis can provide evidence that mutagens reach these germ cells and hence all germ cell stages within the tubules of the testes. Much of the damage detected is unlikely to be transmitted. The test is therefore not appropriate for estimating risk of heritable effects.

7.34.2 *Cytogenetic analysis of meiotic and post-meiotic cells for micronuclei.* This test is applicable in principle for any pre-meiotic stage, but damage might be detected only following treatment of spermatocytes. Positive results suggest that heritable damage is being induced.

7.34.3 *Cytogenetic studies for reciprocal translocations using spermatocytes.* This assay is most commonly applied for detecting genetic damage induced in treated spermatogonial stem cells but it can detect some aberrations induced in primary spermatocytes. 'Reciprocal translocations', which are heritable, are the category of damage assayed. The test can therefore be used for risk assessment with allowance made for post-meiotic loss (50%). Few chemicals so far investigated have proven capable of inducing translocations in stem cells. This could reflect the repair capability of this rather special type of cell.

7.34.4 *The dominant lethal assay.* This screens for genetic damage which is lethal to the zygote. Dominant lethals principally comprise chromosome aberrations, but lethal gene mutations may also be represented. The test is valuable for screening for effects on germ cells since, in the male, effects at all stages of germ cell maturation can be investigated. Such effects are likely to be associated with heritable genetic damage. Furthermore, although the end-point measured is death of the conceptus (as a blastoma or soon afterwards), genetic damage is transferred from F_0 to the F_1 stage. Dominant lethal induction in male post-meiotic germ cells generally indicates that chromosome damage, such as translocations and point (gene) mutation, is being produced and this can also be true with meiotic cells. Dominant lethals resulting from spermatogonial treatment are attributable to the unbalanced product of reciprocal translocations. The occurrence of a sterile period caused by spermatogonial killing sometimes indicates that translocations and/or point mutations are being induced. The test can be applied to females, but interpretation of the results is more difficult due to the systemic effects of the compound. The dominant lethal test has a low sensitivity and fails to detect many somatic cell mutagens with carcinogenic potential, but its prime function is to detect germ cell mutagens.

7.34.5 *The 'one-cell' embryo test.* This method screens for structural and numerical chromosome changes that are induced in parental germ cells and visualised in the embryo. Chromatid aberrations are detected following the treatment of oocytes or male post-meiotic germ cells with chemicals that require a round of DNA synthesis to express damage. Certain types of chromosome aberrations are detected with chemicals that induce chromosome damage directly. The test generally serves the same function as the dominant lethal test.

7.34.6 *Non-disjunction test in ten-day mouse embryo.* It is possible to investigate non-disjunction in the mouse by cytological examination of 10-day-old embryos for trisomy after parental treatment. The method is, however, very difficult, requiring considerable technical expertise. The more direct approach of screening metaphase II cells of either sex is less demanding and the results may be extrapolated directly to man. Genetic methods requiring less technical expertise are becoming available.

7.34.7 *The mouse specific locus (and dominant visible) test.* This screens for point mutations, some of which can be small deletions. It can be applied to any germ cell stage but is only really appropriate for spermatogonial stem cells. Because of the large numbers of animals needed, the test is justified only in exceptional circumstances, when a full risk-benefit analysis is necessary.

7.34.8 *The mouse heritable translocation test.* This may be applied to all germ cells. It is most effective for male post-meiotic stages, but has been used to detect low levels of effect in spermatogonial stem cells. Because it is labour-intensive and requires large numbers of animals its use is justified only when a full risk-benefit analysis is required.

7.34.9 *The sperm morphology assay.* This assay has, in addition, been investigated as a mutagenicity test, but the results in some circumstances have been due to direct toxicity or malnutrition rather than to mutagenicity, or from a combination of both.

Summary

7.35 An outline recommended testing strategy is given in Figure 7.1.

7.36 This strategy must not be regarded as an inflexible step-sequence of testing to be rigidly followed in all cases. A flexible approach should be adopted, with each compound being considered on a case-by-case basis, taking into account: (i) the chemical structure of the compound [information on the reactivity of the substance or its metabolites and the general chemical class (eg, azo-dyestuff), can be very useful]; (ii) information on absorption, metabolism and distribution; (iii) the existing knowledge of its mutagenic properties; and (iv) its intended use and the resulting exposure to man.

7.37 Thus, Stage 1 covers the type of tests that should be carried out for all new chemicals and products. If for any reason suspicion arises about any chemical already in wide use, it should also be subjected to these testing procedures. Where a chemical is not proposed for human ingestion, and where it is expected that its use will be limited, and the degree of human exposure small or containable, it is acceptable to limit investigation to two tests, one to detect gene mutation in bacteria, and the second to detect clastogenic effects in mammalian cells. Where it is expected that human exposure, although at low dose levels, will be sustained and ubiquitous (eg, food additives, pesticides, cosmetic preparations or domestic chemicals), the third test is also necessary.

7.38 The need to carry out these studies to a high quality, and to fulfil rigorous protocols, should be emphasised. The size of the study should be adequate to obtain significant results. The emphasis is on a limited number of *in vitro* studies carried out to a very high standard.

7.39 The Stage 2 studies cover *in vivo* investigations of mutagenic potential in somatic cells. It is not justifiable to use such studies for general screening purposes, since the three *in vitro* studies in Stage 1 should detect essentially all compounds possessing mutagenic potential. For most compounds *in vivo* testing would be necessary only if mutagenicity was demonstrated *in vitro* and there was thus a need to show if this potential could be demonstrated *in vivo* and to enable a realistic estimate of the likely risk to be made. There was thus a need to show if this potential could be demonstrated *in vivo*. However, in cases where humans were likely to be exposed to high levels of the substance, or to moderate levels for prolonged periods, for example most medicines, certain food additives, or where structural or other indicators suggest concern, the reassurance afforded by *in vivo* testing will be necessary.

Figure 7.1: Flow diagram for testing strategy for investigating mutagenic properties of a substance*

STAGE 1

Initial Screening

Two tests required (a + b) except where human exposure would be expected to be extensive and/or sustained, and, difficult to avoid, when all three tests are necessary

In Vitro Tests

(a) Bacterial assay for gene mutation

(b) Test for clastogenicity in mammalian cells for example metaphase analysis

(c) Test for gene mutation in mammalian cells (for example the L5178Y TK+/− assay)

STAGE 2

Tests for:
Compounds positive in one or more tests in Stage 1,
and
All compounds where high, or moderate, prolonged levels of human exposure are anticipated

In Vivo Tests

(a) Bone marrow assay for chromosome damage (metaphase analysis or micronucleus test)

Plus, if above negative, and any *in vitro* test positive

(b) Test(s) to examine whether mutagenicity or evidence of DNA damage can be demonstrated in other organs (eg liver, gut etc)

STAGE 3

If risk assessment for germ cell effects is justified (on basis of properties including pharmacokinetics, use and anticipated exposure).

Quantitative studies need strong justification in view of their complexity, long duration, costs and use of large numbers of animals.

In Vivo Tests for Germ Cell Effects

(a) Tests to show interaction with DNA

Dominant lethal assay (most useful)

Cytogenetics in spermatogonia

One cell embryo test

(b) Tests to show potential for inherited effects

Dominant lethal assay gives indication of likelihood of inherited effects

Cytogenetics in spermatocytes for reciprocal translocations

Non-disjunction in the mouse (10-day embryo)

(c) Test for quantitative assessment of heritable effects

Mouse heritable translocation test

Mouse specific locus test.

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* General guidance only is given in this flow diagram. Decisions regarding, for example, whether a specific compound is expected to produce high or moderate, but prolonged, exposure would normally be taken by Regulatory Authorities having regard to other relevant data, and on a case-by-case basis.

There may be instances where alternative tests to those specified might be more appropriate, and it is important that a flexible approach is adopted. Each compound should be considered on a case-by-case basis with regard to the selection of tests as well as the interpretation of results.

7.40 The implications of mutagenicity for carcinogensis were considered in Chapter 4. They lead to the prudent assumption that compounds mutagenic *in vivo* in mammals are also potential carcinogens. The testing strategy outlined in Stages 1 and 2 of Figure 7.1 to obtain data on a compound's inherent mutagenic potential from *in vitro* studies, and then to determine whether it has the ability to express this activity *in vivo*, is thus also appropriate for obtaining information on the carcinogenic potential of a substance. Mutagenicity tests are considerably less time consuming and less costly than conventional carcinogenicity bioassays. They can thus be recommended as a practical approach to screening for carcinogenic potential due to mutagenic action. It should be noted, however, that these studies provide very little data on carcinogenic potency or on tissue specificity, and will not detect all potential carcinogens, since some chemical carcinogens appear to act by different (non-mutagenic) mechanisms.

7.41 In addition to the mutagenicity studies described in the earlier sections, cell transformation assays have been proposed by some as being closer models for carcinogenicity, although the relation between the changes observed in the cell systems *in vitro* and malignancy *in vivo* is not understood, and a mechanistic link has not been clearly established. A number of mammalian cell transformation assays have been developed, based in the main on measuring the ability of a substance to induce morphological changes of cells or colonies in cell culture. These changes are thought to be related to malignant transformation *in vivo*, although the precise relevance of the *in vitro* transformed cell phenotype to *in vivo* carcinogenesis is unclear.

7.42 Although it is clear that cell transformation assays have not reached the state of maturity of the assays recommended for screening for mutagenicity mentioned earlier in this chapter, they have unique features that warrant further investigation. Technical aspects of the assays need to be considered to improve their reproducibility and their robustness. There is also a need to investigate further how the cell changes measured relate to the mechanism of carcinogenesis and promotion, and possibly also to carcinogenic potency.

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Appendices

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Appendix 2: Glossary

ADDUCT A chemical moiety which has become covalenty bound to a large molecule such as DNA or protein; the term may cover both the chemical and the portion of the macromolecule with which it is combined.

ALKYLATING AGENT Chemicals which are electrophilic reactants that, without the need for metabolic activation, leave an alkyl group covalently bound mainly to sulphur, nitrogen and oxygen atoms in biologically important macromolecules such as proteins and nucleic acids. They are classified as mono-, bi- and polyfunctional depending on the number of reactive groups they contain. Many alkyating agents are mutagenic, carcinogenic and immunosuppressive.

ALLELES Alleles are alternative forms of a gene found at the same locus on homologous chromosomes.

AMINO ACIDS Compounds of the general formula R-C-NH_2 which are the building blocks of proteins. Many hundreds are known but only 20 are normally found in proteins.

AMNIOCENTESIS Aspiration of amniotic fluid with a needle inserted into the gravid uterus through the abdominal wall. This process allows the examination of fetal cells contained in the amniotic fluid.

ANALOGUE Used in the restricted sense as a compound which is structurally related to another compound.

ANEUPLOIDY A condition in which the chromosomes, number of cells of individuals is not an exact multiple of the typical haploid set for the species. When a single extra chromosome is added to a basically diploid set of two (or more) homologues, the complement is said to be trisomic for that chromosome, the addition of two or more copies of a homologue result in tetrasomy or polysomy. If one chromosome of a pair is missing, the result is monosomy of that particular chromosome (absence of the pair is called nullisomy). It is customary to describe cells of individuals with extra or missing chromosomes as hyperloid or hypoploid respectively.

ANTICODON The three adjacent nucleotides in a transfer-RNA molecule that are complementary to, and base pair with, the three complementary nucelotides of a codon in a messenger-RNA molecule during protein synthesis.

BASE PAIR SUBSTITUTION The substitution of one base for another in a DNA sequence that may lead to an altered protein. There are two kinds of base pair substitution: transitions (one purine is substituted by the other, or one pyrimidine by the other): transversions (a purine is substituted by a pyrimidine or vice versa).

CATALYSE To change the rate of a chemical reaction by means of a substance (a catalyst) that is not itself altered or consumed in the course of the reaction.

CELL LINE An established cell line consists of cultured cells (usually of mammalian origin in this context) which are capable of stable growth for many generations.

CENTROMERE The characteristic region of each chromosome with which the spindle fibre becomes associated during cell division. When a chromosome has replicated it consists of two chromatids joined together at the centromere.

CHROMATID One of the two visibly distinct daughter sub-units of the replicated chromosome.

CHROMOSOME In prokaryotes the circular DNA molecule containing the entire genetic material of the cell. In eukaryotes one of the threadlike structures in the nucleus carrying the genetic information arranged in a linear sequence.

CLASTOGEN An agent (which may be physical, chemical or viral) that produces chromosome breaks and other aberrations.

CODON A group of three adjacent nucleotides in a messenger RNA (mRNA) molecule that codes either a specific amino acid or for polypeptide chain termination. The term is also used for the corresponding sequence in the DNA.

CONGENITAL Present at birth but not necessarily genetic: applied particularly to structural and fuctional defects or diseases.

COVALENT BINDING Bonding formed by the sharing of an electron pair between two atoms. Molecules are combinations of atoms bound together by covalent bonds.

DELETION Usually a chromosome aberration in which a proportion of a chromosome is lost. May also refer to loss of any DNA segment.

DIMER A chemical entity consisting of an association of two monomeric sub-units. The condensation product of either two identical or two similar molecules. As an example pyrimidine dimers are a product of UV irradiation of DNA.

DIPLOID Carrying two homologous sets of chromosomes, the normal state in cells of higher eukaryotes (including humans) except for spermatozoa or ova and their immediate precursors (see also haploid).

DOMINANT MUTATION A mutation that is expressed when present on only one chromosome in a heterozygote.

DOMINANT LETHAL MUTATION A dominant mutation that causes death of an early embryo.

DOMINANT VISIBLE MUTATION A dominant mutation that produces a visible phenotype in the heterozygote.

ENDONUCLEASE An enzyme that hydrolyses internal phosphodiester bonds in a polynucleotide.

EXONUCLEASE An enzyme that hydrolyses terminal phosphoester bonds (at 3-prime and 5-prime ends) in a polynucleotide.

EUKARYOTES A class of organisms which, in contrast to prokaryotes (eg bacteria), comprise cells which have a nucleus in which DNA is organised into characteristic sets of chromosomes. This includes all plants except the blue-green algae and all animals. Cell division occurs by the process of mitosis, or, in the case of gamete formation, by meiosis when germ cell precursors undergo reductive division.

FRAME-SHIFT MUTATION A mutation in which one or two bases are added or deleted to the nucleotide sequence of a gene. These have the effect of making the rest of the information in the gene undecipherable by altering the reading frame (genes are 'read' in groups of three bases, each group constituting a codon).

GAMETE A reproductive cell (ovum or sperm) which normally contains a haploid number of chromosomes.

GENE A functional unit of inheritance: a specific sequence of bases along the DNA molecule. There are at least three different classes of genes: those which code for polypeptides through transcription into messenger RNA (structural genes), those which are transcribed into RNA which is not translated into protein (transfer RNA and ribosomal RNA) and those whose function may not require transcription.

GENE AMPLICATION The process by which the number of copies of a specific gene within a cell can be increased. This usually occurs only when the cell obtains a selective advantage for example where increasing the copy-number of a gene which can increase the rate of cell proliferation. Some oncogenes are amplified during the late stages of cell transformation.

GENOME The sum total of the genes of an organism. In eukaryotes the basic genome is equivalent to the haploid set of chromosomes.

GENOTOXIC Genotoxic is a rather loose term that in a broad sense may refer to the property of a substance as being harmful to genetic material. In Chapter 3 it is used in a narrow sense to refer to the ability of a substance to react with DNA either directly or after metabolic activation.

GENOTYPE The genetic constitution of an individual.

GLYCOSYLASE An enzyme which recognises and removes physically or chemically modified bases (eg alkyl purines) from the sugar phospate DNA chain leaving behind a hole (an abasic site).

HAPLOID The cells of an individual, including the primordial germ cells, possess two sets of chromosomes, one derived from each parent, and are therefore diploid. In gametogenesis the germ cells undergo meiotic division, whereby the double set of chromosomes is reduced to one set (haploid state) in the gametes.

HETEROZYGOTE Where different alleles of a gene are present on each of the two homologous chromosomes.

HISTONES Basic proteins complexed with DNA in the chromosomes of eukaryotes.

HOMOLOGOUS CHROMOSOMES Chromosomes that contain the same linear sequence of genes and as a consequence each gene is present in the genome in duplicate. The chromosome that pair during meiosis are homologous, each homologue being a duplicate of one of the chromosomes from the mother and one from the father.

HYBRID Offspring of animals or plants derived from different inbred lines (or different species).

HYBRIDISATION Hybridisation in its most general sense is to produce hybrids, but the term has also acquired a specialised meaning as a technique for assessing the extent of sequence homology between single strands of nucleic acids for example, *in situ* hybridisation.

HYDROPHOBIC Description term for the tendency of a group of atoms or of a surface to resist becoming wetted of solvated by water.

INITIATION The first step in the carcinogenic process whereby a single cell, or possibly a small number of cells, sustains one or more somatic mutations either spontaneously or as a result of exposure to carcinogens which may be chemical, viral or physical in nature. The critical changes produced in the genome are non-lethal and are inherited in subsequent cell generations.

ISOCHROMOSOME A chromosome aberration in which one of the arms of a particular chromosome is duplicated because the centromere divides transversely and not longitudinally during cell division.

KARYOTYPE The chromosome complement of a cell. The term is used for a photomicrograph in which the chromosomes of an individual are arranged in a standard manner.

LESION A general term indicating damage or injury to the structure and/or function of cells and tissues. Biochemical lesions may result from a defect in, for example, an enzyme system. A wide variety of lesions may be inflicted on DNA molecules by chemicals, viruses and physical agents.

MACROMOLECULE A high molecular weight molecule such as a polymer.

MEIOSIS The special type of cell division occurring in germ cells by which gametes containing the haploid chromosome number are produced from diploid cells. Two meiotic divisions occur, meiosis I and meiosis II. Reduction in chromosome number takes place during meiosis I.

METABOLIC ACTIVATION Conversion, by enzymes, of a chemical from one state to another, for example by hydroxylation, epoxidation or conjugation. The term metabolic activation system is used in a more narrow sense to describe the addition of a preparation of a mammaliam cell-free preparation (usually the 9000 g post mitochondrial supernatent—'S-9') from livers of rats pretreated with a substance which stimulates production of metabolising enzymes. S-9 preparations are added to bacterial and eukaryotic *in vitro* short-term tests to mimic the metabolic activation typical of mammals.

METAPHASE II The metaphase produced in the second division of meiosis, the process in mammals by which four spermatids or ootids are formed (see meiosis).

METHYLATION The introduction of methyl group (CH_3-) into a chemical.

MITOSIS The type of cell division which occurs in somatic cells when they proliferate. Each daughter cell has the same complement as the parent cell.

MONOSOMY A condition in which one chromosome of a pair is missing. An example is monosomy X (45X. Turner syndrome).

MOSAIC An individual or tissue with at least two cell lines differing in genotype or karyotype, derived from a single zygote.

MUTATION A permanent change in the amount or structure of the genetic material in an organism, resulting in a change in the phenotypic characteristics of the organism. The alterations may involve a single gene, a block of genes, or a whole chromosome. Effects involving single genes may be a consequence of effects on single DNA bases (point mutations) or of large changes, including deletions, within the gene. Effects on whole chromosomes may involve numerical or structural changes. A mutation in the germ cells in sexually reproducing organisms may be transmitted to the offspring. A mutation that occurs in somatic cells may be transferred to descendent daughter cells and not to offspring.

MUTATION RATE The rate at which mutations occur at a given locus, expressed as mutations per gamete per locus per generation.

MULTIFACTORIAL Determined by many different factors; it is a term frequently applied to the carcinogenic process.

MULTISTAGE CARCINOGENESIS The development of tumours is regarded as a multistage process in which at least three separate, sequential processes-initiation, promotion and progression-have been described. Much of the evidence is derived from experimentally induced tumours in animals. Direct evidence for multistage carcinogens in man is limited but enough information is available to conclude that several types of human cancer are likely to develop in this manner.

NON-DISJUNCTION At meiosis: the failure of two members of a chromosome pair to disjoin (ie separate) during cell division so that both pass to one daughter cell. At mitosis: the failure of separation of sister chromatids of a chromosome so that both pass to one daughter cell.

NUCLEOPHILE An atom or a group of atoms that tends to donate an electron pair to an electron-deficient region of a molecule.

NUCLEOTIDE The basic unit of a nucleic acid structure, composed of three distinct parts that are joined by convalent-bonds—a pentose sugar (deoxyribose in DNA and ribose in RNA), a nitrogenous base of either purine or pyrimidine derivation, and a phosphate group.

ONCOGENE The name given to activated forms of proto-oncogenes. These can be either viral (v-onc) or cellular (c-onc) in origin. Their names generally derive from the original source of isolation, for example v-ras from rat sarcoma virus, v-myc from myelocytomatosis virus, v-abl from Abelson murine leukaemia virus etc.

OOCYTE The precurser cells of the unfertilised egg or ovum: the latter are produced following meiosis of the oocytes.

PARASEXUAL Reproduction without the involvement of a sexual cycle.

PENETRANCE The frequency (in per cent) with which an allele whether present as a dominant or in a recessive homozygous state, is expressed in the phenotype of an individual.

PEPTIDE; PROTEIN A chain of amino acids joined together by amide linkages formed by condensation of an amino group in one amino acid with the carboxylic acid group in another. The distinction between a peptide and protein is rather arbitary. Naturally occurring peptides have short, flexible chains; proteins have very long chains which are coiled and folded in specific ways which depend on the sequence of amino acids.

PHARMACOKINETICS Pharmacokinetics is the scientific discipline that deals with the mathematical description of rates of absorption, distribution, metabolism and excretion of chemicals.

PHENOCOPY A mimic of a phenotype that is usually determined by a specific genotype, produced instead by some environmental factor.

PHENOTYPE The observable physical, biochemical and physiological characteristic of an organism as determined by its genotype, and the environment in which it develops. In a restricted sense, the expression of some particular gene or genes.

POLAR BODIES The smaller cells that are produced during meiosis in oogenesis and that do not develop into functional ova.

POLYMER A high molecular weight compound consisting of long chains that may be open, closed, linear, branched or cross-linked. The chains are composed of repeating units called monomers which may be identical or different.

POLYMERASE Enzymes that assemble a number of similar or identical subunits into a larger unit or polymer, for example, DNA polymerase or RNA polymerase; enzymes which catalyse the ordered assembly or mononucletodies to reproduce DNA or RNA. They use one of the two strands of the DNA duplex as a template in the process of genetic transcription to RNA (RNA polymerase) or DNA replication or repair (DNA polymerase). RNA may also act as a template for the generation of DNA (reverse trancriptase).

POLYMORPHISM The occurrence in a population of two or more genetically determined alleles in such frequency that the rarest of them could not be maintained by mutation alone.

POST TRANSCRIPTIONAL STABILIZATION The mechanism by which the stability of a messenger RNA species within the cytoplasm of a cell can be maintained or increased. The mechanism is thought to involve protection from attack by specific ribonucleases. This provides a means of producing more gene product within the cell without increasing the rate of transcription.

PROBE A labelled DNA or RNA fragment which hybridises with, and thereby detects, complementary sequences in isolated DNA or RNA, or in intact cells of tissues.

PROGRESSION Easily the least understood phrase in the carcinogenic process whereby benign tumours acquire one or both of the pathognomonic features of malignant growth, the capacity to invade local tissues and to disseminate to distant sites (metastasis). The status of progression is particularly difficult to appraise in human tumours where, at many sites, tumours are malignant *de novo* and do not evolve from an intermediate neoplasm.

PROKARYOTES The simplest living organisms namely viruses, bacteria and some blue green algae, in which the genetic material is arranged into one chromosomal complex consisting of a single circular molecule of DNA (or RNA in some viruses). They lack an organised nucleus. Mitosis and meiosis do not occur, although nucleotide polymerisation (chromonemal) replication takes place and division and multiplication follow.

PROMOTION (TUMOUR) The ill-understood and protracted process whereby initiated cells undergo clonal expansion to form visible tumours. The mechanisms involved are diverse but, in different contexts, include sustained tissue damage with cycles of cell distribution and regeneration, immune suppression, hormonal imbalance, alterations in intercellular contacts and release of cells from normal growth control. Specific chemical promoting agents have been characterised (notably the phorbol esters) but their mode of action is unclear except that, in general terms, chemical promoters are not directly mutagenic.

PROTO-ONCOGENE A group of normal cellular genes which are highly conserved and probably concerned with cell proliferation. They can, however, be activated in various ways to forms which are closely associated with one or more steps in cell transformation. Mechanisms of activation include point mutations which alter the structure of the proto-oncogene or changes in the regulatory regions which alter the level of expression. Activating agents include viruses and chemicals. The process of proto-oncogene activation is thought to play an important part in the development of tumours.

PURINE A basic, heterocyclic, two-ringed nitrogen containing compound that occurs in nucleic acids. Common purines are adenine and guanine.

PYRIMIDINE A basic heterocyclic, one-ringed nitrogen containing compound that occurs in nucleic acids. Common pyrimidines are cytosine, thymidine and uracil.

PYRIMIDINE DIMER See dimer.

RECESSIVE MUTATION A mutation which is expressed only when carried by both homologous chromosomes in a diploid organism (ie, not in a heterozygote).

RETROVIRUS A mammalian virus that has RNA as its genetic material and which undergoes reverse transcription to copy its RNA sequences into DNA which is subsequently integrated into the host genome. Some retroviruses acquire cellular genes and can become acutely transforming retroviruses which rapidly induce tumours in some laboratory animals. Most retroviruses are not of this type however, and there are, in fact, retrovirus-like sequences integrated into the genomes of all mammalian cells, including human cells.

RIBOSOMES Intracellular organelles present in the cytoplasm of all mammalian cells and in the cells of other organisms (fungi, bacteria etc). They consist of two sub-units each composed of RNA and protein, and participate in the synthesis of polypeptides whose amino acid sequences are specified by the nucleotide sequences of messenger RNA molecules.

ROBERTSONIAN TRANSLOCATIONS A translocation between two acrocentric chromosomes by fusion at the centre and loss of the short arms.

SPERMATOGONIAL STEM CELLS Self-renewing component of the spermatogonial population of male gonads from which all the other male germ cells derive. May be quiescent or have long cell cycle unless depleted by agents that kill cells. Mutations induced in this cell type are maintained and transmitted to the progeny throughout the lifetime of the individual.

SPERMATOGONIA (DIFFERENTIATED) The series of mitotically-active cells of the male gonads derived from the stem cells which give rise to primary spermatocytes.

SPERMATOCYTES Those cells of the male gonads that undergo meiosis to form spermatids. In the first of the two meiotic divisions, a primary spermatocyte (diploid) gives rise to two secondary spermatocytes. These in turn divide into four spermatids (haploid).

SPERMATIDS These are cells formed following, or by, meiosis in the male gonads. They undergo a process of maturation without further division, known as spermiogenesis, to produce spermatozoa.

SPINDLE INHIBITOR A substance that inhibits the formation of spindle fibres, that is, the collection of microtubule filaments which are responsible for the movement of eukaryotic chromosomes during nuclear division. Spindle inhibitors thus delay mitosis. An example is colchicine.

TAUTOMERISM An equilibrium between two isometric forms of a molecule that differ significantly in both the relative positions of the atoms and in the bonds between the atoms; the molecules may react in either of the two isomeric forms depending on the conditions.

TOXIN A general term for a chemical agent, often of biological origin (from bacteria, plants etc), which can cause injury or disease.

TRANSDUCTION The name given to the process by which a virus ‘picks up’ a cellular gene during intracellular passage. In the case of a replication—competent retrovirus, the process involves multiple recombination events and usually results in the production of a replication-defective retrovirus in which some of the genetic information is replaced by a cellular gene. The acutely transforming retroviruses which led to the discovery of oncogenes evolved in this way.

TRANSCRIPTION The process by which the genetic message carried in the base sequence of DNA is transferred to RNA (messenger RNA) by complementary base-pairing of ribonucleotide triphosphates with DNA bases.

TRANSFECTION A process by which DNA is introduced into living cells, typically in the course of a viral infection. Transfection can be performed experimentally where it is increasingly used to study the function of cloned genes (eg, for the detection of activated oncogenes in high molecular weight DNA from tumour samples).

TRANSFORMATION The process by which a normal cell acquires the capacity for neoplastic growth. Complete transformation occurs in several stages both *in vitro* and *in vivo*. One step which has been identified *in vitro* is 'immortalization' by which a cell acquires the ability to divide indefinitely in culture without undergoing senescence. Such cells do not have the capacity to form tumours in animals but can be induced to do so by extended passage *in vitro* or by treatment with chemicals or transfection with oncogene DNA. The transformed phenotype so generated is usually, but not always, associated with the ability of the cells to grow in soft agar and to form tumours in syngeneic animals. It should be noted that each of these stages of transformation can involve multiple events which might or might not be genetic. The order in which these events take place, if they occur at all, *in vivo*, is not known.

TRANSLOCATION The transfer of a region of one chromosome to another chromosome. If two non-homologous chromosome exchange pieces, the translocation is reciprocal.

TRISOMY The state of having three representatives of a given chromosome instead of the usual pair, as in trisomy 21 (Down syndrome).

TRIPLOID A cell with three complete basic sets of chromosomes (ie, three of each homologue).

TUMOUR SUPPRESSOR GENE A gene whose continued expression is thought to be essential for normal growth and differentiation of cells. Many tumour suppressor genes probably exist. Deletion or suppression of such genes appears to favour development (see Chapter 3, page 8 for a more complete explanation).

ULTIMATE MUTAGEN The name given to the active form (usually a metabolite) of a mutagen which binds to the genetic material and forms an adduct.

UNSCHEDULED DNA SYNTHESIS (UDS) DNA synethesis that occurs at some stage in the cell cycle other than the S period (the normal or 'scheduled' DNA synthesis period) in response to DNA damage. It is usually associated with DNA repair.

X-INACTIVATION Genetic inactivation of genes on one of the two x-chromosome in early embryonic life in the somatic cells of mammalian females.

Appendix 3: Terms of reference for the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment

To advise, at the request of:

Department of Health
Ministry of Agriculture, Fisheries and Food
Department of the Environment
Department of Trade and Industry
Department of Transport
Department of Energy
Health and Safety Executive
Medicines Commission, Section 4 Committees and the Licensing Authority
Committee on the Medical Aspects of Food Policy
Home Office
Scottish Home and Health Department
Department of Agriculture and Fisheries for Scotland
Welsh Office
Department of Health and Social Services for Northern Ireland
Other Government Departments

I. To assess and advise on the mutagenic risk to man of substances which are:—

a. used or proposed to be used as food additives, or used in such a way that they might contaminate food through their use or natural occurrence in agriculture, including horticulture and veterinary practice or in the distribution, storage, preparation, processing or packaging of food,

b. used or proposed to be used or manufactured or produced in industry, agriculture, food storage or any other workplace,

c. used or proposed to be used as household goods or toilet goods and preparations,

d. used or proposed to be used as drugs, when advice is requested by the Medicines Commission, Section 4 Committees or the Licensing Authority,

e. used or proposed to be used or disposed of in such a way as to result in pollution of the environment.

II. To advise on important general principles or new scientific discoveries in connection with mutagenic risks, to co-ordinate with other bodies concerned with the assessment of mutagenic risks and to present recommendations for mutagenicity testing.

Note:
The Committee considers that the term mutagenicity as used in its terms of reference should be used in the same sense as it is used by, for example, the

EEC and include short-term tests of carcinogenicity such as the cell transformation assay and also any other tests designed to assess the effect of a chemical on any part of the genetic apparatus of the cell.

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